Glyphosate &
the IPA-, K-, NH_4 - and DMA salts of glyphosate

Herbicide

Application for Renewal of Approval (AIR 2) according to Commission Regulation (EC) N° 1141/2010

ANNEX II

Document M:

Point 8: Ecotoxicological studies on the active substance

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IIA 8 Ecotoxicological studies on the active substance

IIA 8.1 Avian toxicity

An extensive regulatory avian toxicology database has been summarized to evaluate acute, short-term and long term toxicity of glyphosate, glyphosate salts, AMPA and representative formulation. Tier 2 summaries for new studies previously not evaluated during the 2001 EU evaluation of glyphosate have been included. The results from all of these studies demonstrate that glyphosate, glyphosate salts, AMPA and glyphosate formulations are of low acute, short-term and long-term toxicity to birds.

The conclusion of the acute and short-term assessments in the 2001 EU evaluation of glyphosate stated that, glyphosate, AMPA and glyphosate formulations are of low acute oral toxicity to birds and do not pose an unacceptable acceptable risk to birds. Additionally, it was concluded that long-term risk to birds is regarded to be acceptable with TER values greater than the acute, short-term and long-term triggers. For the long-term assessment, two additional one-generation reproduction studies conducted with mallard duck and bobwhite quail have been summarized and confirm the results of the older studies demonstrating low long-term toxicity of glyphosate to birds.

A summary of all available relevant and compliant data (including data already reviewed during the 2001 EU evaluation of glyphosate) for glyphosate, glyphosate salts and AMPA is presented in Table 8.1-1 and Table 8.1-3.

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Table 8.1-1: Acute toxicity of glyphosate acid, glyphosate-IPA salt, glyphosate K-salt and AMPA to birds. Values in bold are the EU endpoints for the 2001 EU evaluation of glyphosate.

Species	LD_{50}/LC_{50} (mg a.s./kg bw)	NOEC (mg a.s./kg b.w.)	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid				
Bobwhite quail	> 3851	1785	139-140 1978/no[1]	B 8.1.1 AVS93-00041
Bobwhite quail	> 2000	2000	IIA 8.1.1/01 400/963858 1997/yes	NA
Bobwhite quail	**> 2000**	2000	48/91266 1991/yes	B 8.1.1 AVS94-00230
Japanese quail	> 2000	2000	IIA 8.1.1./02 1413/4-1011 1996/yes	NA
Japanese quail	> 2000	2000	IIA 8.1.1/03 D8.1–382/99 1999/no	NA
Mallard duck	> 2000	2000	IIA 8.1.1./04 1413/5-1011 1996/yes	NA
Mallard duck	**> 2000**	2000	49/91843 1992/yes	B 8.1.1 AVS94-00229
Glyphosate-IPA salt				
Japanese quail	>1225.4 [2]	1225.4	-D81.186/00 , 2000/yes	NA
Glyphosate K-salt				

Bobwhite quail	>2241	484	IIA 8.1.1./05 [redacted] 2002-151 [redacted] 2003/yes	NA
AMPA				
Bobwhite quail	**> 2250**	1350 [3]	139-277 [redacted] 1991/yes	B 8.1.1 AVS95-00222

[1] Study was performed with 14 d aged birds rather than > 16 w old specimens; sex was not determined due to age of birds. NOEL value is based on the observation of transient lethargy in the 3851 mg/kg bw dose group on the day of dosing. No other adverse effects were observed at 3851 mg/kg bw. This study was concluded to be valid in the 2001 EU evaluation of glyphosate

[2] Limit test; test was conducted with 2000 mg glyphosate IPA salt/kg bw (nominal), equivalent to 1225.4 mg glyphosate/kg bw.

[3] This NOEL value is based on the observation of transient lower limb weakness, a ruffled appearance, and reduced reaction to external stimuli (sound and movement) on the day of dosing.

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)
NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

Only Tier II summaries are presented for those studies that were not reviewed during the 2001 EU Glyphosate evaluation, with the exception of two long-term avian studies, and that meet current quality and guideline criteria.

As no mortality was observed in any of the acute limit dose studies listed above, the relevant acute endpoint for exposure of birds to glyphosate acid was determined by extrapolation of the acute LD_{50} of >2000 mg a.s./kg bw. According to the current EFSA Guidance Document for birds and mammals, an acute extrapolation factor of 2.167 may be applied when at least 20 individuals are tested at limit dose. Considering a limit dose of 2000 mg a.s./kg bw and the fact that 50 birds were tested for glyphosate acid, a **LD_{50} = 4334 mg a.s./kg bw** was extrapolated.

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Table 8.1-2: Short-term toxicity of glyphosate acid and the metabolite AMPA to birds. Values in bold are the EU endpoints for the 2001 EU evaluation of glyphosate.

Species	NOEC/ NOAEC	LC_{50}	LC_{50}	NOEC/ NOAEC	Reference/ GLP	2001 EU evaluation monograph reference
	(mg a.s./kg feed)		(mg a.s./kg bw/day) [1]			
Glyphosate acid						
Bobwhite quail	4640 [2]	**>4640**	>1127	1127	73-76 1973/no	B 8.1.2 AVS93-00043
Bobwhite quail	5200[2]	>5200	>1511	1511	395/963857 1997/yes	NA
Japanese quail	5000[2]	>5000	- [3]	- [3]	1085 1989/yes	B 8.1.2 AVS95-00158
Mallard duck	4640 [2]	**>4640**	>1242	1242	73-15 1973/no	B 8.1.2 AVS93-00042
Mallard duck	5200[2]	>5200	>1715	1715	23 1997/yes	NA
AMPA						
Bobwhite quail	5620 [2]	**>5620**	>2162	2162	90-398 1991/yes	B 8.1.2 AVS95-00220
Mallard duck	5620 [2]	**>5620**	>1765	1765	90-399 1991/yes	B 8.1.2 AVS95-00221

[1] Calculation described in Appendix I
[2] Highest dose tested.
[3] study provided insufficient body weight data to permit calculation of a daily dose

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph
NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

The studies on short-term toxicity of glyphosate acid and AMPA demonstrate low toxicity. According to the 'Guidance of EFSA, Risk Assessment for Birds and Mammals', European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2009: 7(12):1438, a risk assessment of short term toxicity to birds is only required under specific circumstances and is not foreseen to be required in the glyphosate assessment because of the low acute and long-term toxicity.

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Table 8.1-3: Long-term toxicity of glyphosate acid to birds. The endpoints in bold have been used for the long-term risk assessment

Endpoint	Species	NOEC/ NOAEC (mg a.s./kg feed)	NOEC/ NOAEC (mg a.s./kg bw/day) [1]	Reference/ GLP	2001 EU evaluation monograph reference
Glyphosate acid					
Reproduction one generation, 20 weeks	Bobwhite quail	2250 [2]	**201**	IIA 8.1.4./01 123-186 [redacted] 1999/yes	NA
Reproduction one generation, 21 weeks	Mallard duck	2250 [2]	300	IIA 8.1.4./02 123-187 [redacted] 1999/yes	NA
Reproduction one generation, 17 weeks	Bobwhite quail	1000 [2,3]	96.3	IIA 8.1.4./03 139-141 [redacted] 1978/no	B 8.1.3 AVS93-044
Reproduction one generation, 17 weeks	Mallard duck	1000 [2]	125.3	IIA 8.1.4./04 139-143 [redacted] 1978/no	B 8.1.3 AVS93-045

Endpoints in bold has been selected and used for the long-term risk assessment

1 Calculation described in Appendix I.

2 Highest dose tested.

3 In the original bobwhite quail study was concluded to be 200 mg/kg diet, rather than 1000 mg/kg feed, based on a statistically significant finding that was concluded not to be biologically significant by the study director. Although there was a small reduction in egg weight at 1000 mg/kg feed there was no significant impact on the biologically relevant endpoints of initial hatchling body weight, 14 day hatchling body weight, egg shell thickness and hatchling survival. Egg weight is not a standard endpoint in avian reproduction studies and was a carryover from poultry performance studies. Consequently, the NOAEC concentration was concluded by the Study Director to be 1000 mg a.e./kg diet.

NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

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Tier 2 summaries for all long-term bird studies have been included in this section. The two studies originally reviewed in the in 2001 EU evaluation of glyphosate have been included because a higher endpoint is being proposed based on the availability of new two additional one-generation bird reproduction studies.

In the Glyphosate Monograph, the NOEC for the original bobwhite quail study ([redacted] 1978) was concluded to be 200 mg/kg diet, rather than 1000 mg/kg feed, based on a statistically significant finding that was concluded not to be biologically significant by the study director. Although there was a small reduction in egg weight at 1000 mg/kg feed there was no significant impact on the biologically relevant endpoints of initial hatchling body weight, 14 day hatchling body weight, egg shell thickness and hatchling survival. Egg weight is not a standard endpoint in guideline avian reproduction studies and was a carryover from poultry performance studies. Consequently, the NOAEC concentration for this older glyphosate quail study ([redacted] 1978) is considered to be 1000 mg a.e./kg diet.

Since no adverse effects were observed at the highest concentrations tested in the reproduction studies, the highest NOEC value (**NOEC = 201 mg a.e./kg bw/day** for bobwhite quail) has been used for the long-term risk assessment. This is supported by the current guidance document on Risk Assessment for Birds and Mammals (EFSA 2009), stating that in the case that more than one reproduction study was conducted on the same species, it is possible to merge the data sets as if it were one study. The corresponding study ([redacted] 1999) was not reviewed in the 2001 EU evaluation of glyphosate; however, it has been widely

used to support member state re-registrations and is summarised in Section IIA 8.1.4. These two new one generation reproduction studies confirm the conclusions of the 2001 EU evaluation of glyphosate that glyphosate does not pose unacceptable long-term risk to birds.

IIA 8.1.1 Acute oral toxicity to a quail species (Japanese or Bobwhite), mallard duck or other bird species

Annex point	Author(s)	Year	Study title
IIA 8.1.1/01	[redacted]	1997	Glyphosate acid. Acute oral toxicity (LD_{50}) to Bobwhite quail [redacted] Report No: [redacted] 400/963858 Date: 1997-02-03 GLP: yes not published

Guideline: FIFRA subdivision E, section 71-1

Deviations to OECD 223: None

Dates of experimental work: 1996-12-17 to 1996-12-31

Executive Summary

A laboratory study with the Bobwhite quail (*Colinus virginianus*) was conducted. After an acclimation period of 14 days, birds received a single dose of the test substance glyphosate diluted in methylcellulose (1% w/v) by oral gavage. The test consisted of three dosage groups and a control group. Nominal dosages used in the study were 500, 1000 and 2000 mg a.s./kg bw. The control birds received an equivalent volume of methylcellulose only.

During the test all mortalities and health of the birds were observed daily. Body weights were measured individually 15 and 7 days prior to test start, at the initiation of the test (immediately prior to dosing) and on days 7, and 14 of the test. Feed consumption was determined by cage of each dosage group and the control group 15, 8, 7 and 1 day(s) prior to test start and on days 1 to 7 and 8 to 14 of the test.

Post mortem examination was carried out on all ten control birds and all ten birds from the highest dose group.

There were no mortalities observed in any treatment. All birds remained in good health following dosing, and no clinical signs of toxicity were observed. No treatment-related effects were recorded on body weight and food consumption. No abnormalities were detected in any birds during *post mortem* examination at termination of the study. All validity criteria according to the current guideline OECD 223 were fulfilled.

The acute oral LD_{50} for Bobwhite quail exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid

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Description: White crystalline powder
Lot/Batch #: P24
Purity: 95.6%

2. Vehicle and/or positive control: Methylcellulose (1% w/v) as vehicle and negative control

3. Test organisms:

Species: Bobwhite quail (*Colinus virginianus*)
Age: Young adults, approximately 4 - 6 month old on arrival
Weight: 175 - 213 g (15 days prior to test initiation)
Source: Commercial supplier (UK)
Diet/Food: Standard layer diet in pellet form obtained from UK). Food was offered *ad libitum*, with the exception of an overnight starvation period of approximately 21 hours prior to dosing. Water was available at all times.
Acclimatisation: 15 days

4. Environmental conditions:

Temperature: 17 - 19°C
Relative humidity: 68%
Photoperiod: 10 hours light / 14 hours darkness

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The dose level was based on existing toxicity data indicating that the test material is of low toxicity to birds. Young adult Bobwhite quails (5 males and 5 females per dosage) received a single dose of the test substance or vehicle by oral intubation using a disposable syringe and a Ch 10 Nelaton plastic catheter. The test consisted of three dosage groups and a control group. Nominal dosages used in the study were 500, 1000 and 2000 mg a.s./kg bw (dosage concentrations: 5%, 10% and 20% w/v). A constant dose volume of 10 mL/kg bodyweight was used for all treatment groups. The control birds received an equivalent volume of methylcellulose only.

2. Observations: During the test all mortalities, bird health and clinical signs of the birds were observed daily. Body weights were measured individually 15 and 7 days prior to test start, at the initiation of the test (immediately prior to dosing) and on days 7, and 14 of the test. Feed consumption was determined by cage of each dosage group and the control group 15, 8, 7 and 1 day(s) prior to test start and on days 1 to 7 and 8 to 14 of the test.

Post mortem examination was carried out on all ten control birds and all ten birds from the highest dose group.

3. Statistical calculations: Since no mortality was reported, no statistical calculation of LD_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

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II. RESULTS AND DISCUSSION

A. FINDINGS

The LD_{50} and NOEC values are given below based on nominal doses.

Endpoints	Glyphosate acid [mg a.s./kg bw]
LD_{50}	> 2000
NOEL	2000

Table 8.1.1-1: Effects of glyphosate acid on body weight and food consumption of Bobwhite quail

Glyphosate acid [mg a.s./kg bw]			Control	500	1000	2000
Average body weight per animal [g] (± SD)						
Body weight	Day -15	male	192 ± 5.9	195 ± 5.9	192 ± 3.7	195 ± 4.9
		female	191 ± 11.4	191 ± 15.6	191 ± 13.3	190 ± 8.9
	Day -7	male	196 ± 5.7	196 ± 6.5	194 ± 4.1	198 ± 5.6
		female	190 ± 10.2	190 ± 18.2	192 ± 7.8	189 ± 11.6
	Day 0	male	194 ± 4.7	197 ± 6.9	193 ± 4.8	198 ± 5.9
		female	190 ± 9.1	189 ± 17.1	192 ± 10.6	186 ± 10.5
	Day 7	male	198 ± 2.5	199 ± 6.1	196 ± 4.3	198 ± 8.8
		female	192 ± 13.0	192 ± 18.9	197 ± 13.3	191 ± 9.7
	Day 14	male	200 ± 2.3	199 ± 4.9	196 ± 3.8	196 ± 7.0
		female	192 ± 8.6	194 ± 17.0	198 ± 10.6	189 ± 9.5
Body weight change	Days 0-14	male	6.0 ± 2.4	2.0 ± 2.0	3.0 ± 1.0	-2.0± 1.1
		female	2.0 ± 0.5	5.0 ± 0.1	6.0 ± 0.0	3.0 ± 1.0
Mean food consumption per animal [g/bird/day]						
Food consumption	Day -15 to -8	male	13	13	12	13
		female	13	13	12	13
	Day -7 to -1	male	13	13	12	13
		female	13	13	13	13
	Day 1 to 7	male	14	15	14	13
		female	16	15	15	15
	Day 8 to 14	male	14	14	14	13
		female	15	13	14	14
Group mean	Day 1-14	male	14	14.5	14	13
		female	15.5	14	14.5	14.5

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B. OBSERVATIONS

There were no mortalities observed in any treatment. All control and test birds remained in good health following dosing, and no clinical signs of toxicity were observed. Body weight changes were similar in all groups and there was no evidence of any treatment-related effects. Group mean food consumption was similar in all groups and there was no evidence of any treatment-related effects. No abnormalities were detected in any birds during *post mortem* examination at termination of the study.

All validity criteria according to OECD 223 were fulfilled, as no non-incidental death was observed in the control groups.

III. CONCLUSION

The acute oral LD_{50} for Bobwhite quail exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

Annex point	Author(s)	Year	Study title
IIA 8.1.1/02	[redacted]	1996	Glyphosate: Acute Oral Toxicity to Japanese Quail [redacted] Report No: 1413/4-1011 Date: 1996-07-23 GLP: yes Not published

Guideline: US EPA Guideline, Section E, Series 71.1, Avian single dose oral LD_{50} test (1982).

Deviations to OECD 223: None

Dates of experimental work: 1996-01-09 to 1996-01-23

Executive Summary

A laboratory study was performed to determine the acute oral toxicity of glyphosate acid to Japanese quail (*Coturnix coturnix japonica*). As no mortalities were observed in a range finder study at a maximum limit dose of 2000 mg a.s./kg bw, this dose was tested for the definitive study. Twenty animals were randomly allocated to two groups, one treatment item group and one control, each comprising five males and five females. On Day 0, a single oral dose was administered by direct intubationof2000 mg a.s./kg bw to the treatment item group. The control group was treated with vehicle only (0.5 % w/w CMC solution).

Birds were observed for clinical signs of toxicity, behaviour, body weight effects, food consumption and mortality for 14 days after dosing. Body weights were measured individually at test initiation(day 0), on day 3, 7 and 14 after test initiation. Food consumption for each cage of animals was measured per time interval covering day 0-7, and day 7-14.

No treatment related mortality was observed, except for one bird found dead due to trauma of reproductive tract. Furthermore, there were no effects observed on bodyweight or food intake, and no abnormal findings at necropsy.

All validity criteria according to the current OECD guideline 223 were fulfilled.

The acute oral LD_{50} for Japanese quail exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White/off-white crystalline powder
Lot/Batch #:	H95 D161A
Purity:	95.3%
2. Vehicle and/or positive control:	0.5% carboxymethyl cellulose (CMC) as vehicle and negative control
3. Test organisms:	
Species:	Japanese quail (*Coturnix coturnix japonica*)
Age:	Young adults, approx. 23 weeks old
Weight:	202 - 300 g (at test initiation)
Source:	[redacted] UK
Diet/Food:	Proprietary avian food, *ad libitum*
Acclimation period:	5 weeks prior to dosing
Fasting	16 to 17 hours prior to dosing
4. Environmental conditions:	
Temperature:	15 – 20°C
Humidity:	40 – 78%
Photoperiod:	8 hours light / 16 hours dark

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finder study, an acute oral toxicity test was performed by administering a single limit dose of 2000 mg glyphosate a.s./kg bw (dissolved in 0.5% carboxymethyl cellulose) by oral intubation to ten adult Japanese quails (5 males and 5 females)in one treatment group. In addition, one control group was administered an equivalent volume of the vehicle (CMC) only as the test groups, at a dose rate of 2 mL/kg bw. After dosing, birds where fed *ad libitum* throughout the study.

2. Observations: Birds were caged and observed continuously for signs of toxicity, abnormal behaviour and mortality for one hour after dosing, then at intervals throughout day 0 and twice daily thereafter. Food consumption was measured covering day 0-7, and day 7-14. Each animal was weighed at least on day 0, 3,7 and 14. On day 14, all surviving animals were sacrificed and a gross macroscopic examination was carried out. The necropsy comprised a general inspection of major visceral organs.

3. Statistical calculations: Since the mortality was < 50%, no statistical calculation of LC_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

The LD_{50} value is given below based on nominal doses

The LD_{50} and NOEC values are given below based on nominal doses.

Endpoints	Glyphosate acid [mg a.s./kg bw]
LD_{50}	> 2000
NOEL	2000

Table 8.1.1-2: Effects of glyphosate acid on body weight and food consumption of Japanese quail

Glyphosate acid [mg a.s./kg bw]			Control	2000
Average body weight per animal [g] (± SD)				
Body weight	Day 0	male	249 ± 27.1	228 ± 22.3
		female	257 ± 15.3	260 ± 28.0
	Day 3	male	270 ± 31.4	231 ± 22.2
		female	268 ± 18.5	272 ± 36.1
	Day 7	male	275 ± 31.8	239 ± 17.3
		female	271 ± 18.5	271 ± 32.8
	Day 14	male	276 ± 33.5	243 ± 18.5
		female	276 ± 18.2	288 ± 28.7
Body weight change	Days 0-14	male	26 ± 12	15 ± 5.7
		female	19 ± 13.6	23 ± 3.0
Mean food consumption per animal [g/bird/day]				
Food consumption	Day 0-7	male	64.4	39.9
		female	56.1	60.9
	Day 7-14	male	50.0	41.8
		female	58.0	67.9
Group mean	Day 0-14	mean	57.2	52.0

B. OBSERVATIONS

There was no treatment-related mortality observed, except for one bird in treatment group found dead due to trauma of the reproductive tract. Furthermore, there were no adverse effects were observed on bodyweight or food intake. No findings at necropsy, considered to be treatment-related.

All validity criteria according to OECD 223 were fulfilled, as no non-incidental death was observed in the control groups.

III. CONCLUSION

The acute oral LD_{50} for Japanese quail exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

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Annex point	Author(s)	Year	Study title
IIA 8.1.1/03		1999	Avian Single-Dose Acute Oral Toxicity Test in Japanese Quail with the chemical product Glifosate Técnico Nufarm Report No: D8.1 – 382/99 Date: 1999-12-20 GLP: no not published

Guideline: Not stated

Deviations to OECD 223: None

Dates of experimental work: 1999-10-05 to 1999-10-19

Executive Summary

A laboratory study was performed to determine the acute oral toxicity of glyphosate acid to Japanese quail (*Coturnix coturnix japonica*). Twenty animals were randomly allocated to two groups, one treatment item group and one control, each comprising five males and five females. On Day 0, a single oral dose of 2000 mg glyphosate acid/kg bw was administered enclosed in gelatin capsules. A control group received empty capsules.

Birds were observed for clinical signs of toxicity, behaviour, body weight effects, food consumption and mortality for 15 days after dosing. Birds were weighed at the beginning and at the end of test.

There were no mortalities observed in any treatment group and all birds remained in good health following dosing, with no clinical signs of toxicity were observed. All validity criteria according to the current guideline OECD 223 were fulfilled.

The acute oral LD_{50} for Japanese quail exposed to glyphosate acid was determined to be > 2000 mg a.s. /kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: powder
Lot/Batch #: 037-919-113
Purity: 95% (nominal)
954.9 g/kg acid equivalent (measured)

2. Vehicle and/or positive control: Gelatin capsules as vehicle and negative control

3. Test organisms:

Species: Japanese Quail (*Coturnix coturnix japonica*)

Age: Young adults, at least 16 weeks old

Weight: Males: 100 – 130 g at test start
Females: 114 – 140 g at test start
Source: Not stated
Diet/Food: Commercial diet ([redacted]) and water *ad libitum*.
Acclimatisation: At least 15 days

4. Environmental conditions:

Temperature: 25 - 28°C
Relative humidity: 30 - 70%
Photoperiod: 10 hours light / 14 hours dark

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Young adult Japanese quails (5 males and 5 females per treatment) received a single limit dose of 2000 mg/kg bw of the test substance, enclosed in gelatin capsules. A control group received empty capsules by oral gavage.

2. Observations: During the 15 days of the test, mortality, behaviour, clinical symptoms and anatomopathological alterations were observed daily. Birds were weighed at the beginning and at the end of test.

3. Statistical calculations: Since no mortality was reported, no statistical calculation of LD_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

The LD_{50} and NOEC values are given below based on nominal doses.

Endpoints	Glyphosate acid [mg a.s./kg bw]
LD_{50}	> 2000
NOEL	2000

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Table 8.1.1-3: Effects of glyphosate acid on body weight and food consumption of Japanese quail

Glyphosate acid [mg a.s./kg bw]			Control	2000
Average body weight per animal [g] (± SD)				
Body weight	Day 0	male	109 ± 9.3	123 ± 5.3
		female	121 ± 5.8	116 ± 3.9
	Day 7	male	113 ± 11.1	121 ± 6.9
		female	122 ± 10.2	114 ± 9.9
	Day 14	male	119 ± 9.5	126 ± 6.9
		female	130 ± 9.6	123.8 ± 7.6
Body weight change	Days 0-14	male	10.2 ± 5.0	3.4 ± 5.5
		female	8.8 ± 7.4	7.8 ± 6.3
Mean food consumption per animal [g/bird/day]				
Food consumption	Day 0-7		88.7	99.4
	Day 7-14		77.2	99.6
Group mean	Day 0-14	mean	83.0	99.5

B. OBSERVATIONS

There were no mortalities observed in any treatment. All control and test birds remained in good health following dosing, and no clinical signs of toxicity were observed. Body weight changes were similar in all groups and there was no evidence of any treatment-related effects. Group mean food consumption was similar in all groups and there was no evidence of any treatment-related effects. No abnormalities were detected in any birds during *post mortem* examination at termination of the study.

All validity criteria according to OECD 223 were fulfilled, as no non-incidental death was observed in the control groups.

III. CONCLUSION

The acute oral LD_{50} for Japanese quail exposed to glyphosate acid was determined to be > 2000 mg a.s. /kg bw. The NOEL in the study was determined to be 2000 mg a.s./kg bw.

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Annex point	Author(s)	Year	Study title
IIA 8.1.1./04	[redacted]	1996	Glyphosate: Acute Oral Toxicity to Mallard Duck [redacted] Report No: 1413/5-1011 Date: 1996-07-18 GLP: yes Not published

Guideline: US EPA Guideline, Section E, Series 71.1, Avian single dose oral LD_{50} test (1982)

Deviations to OECD 223: None

Dates of experimental work: 1995-12-14 to 1996-02-18

Executive Summary

A laboratory study was performed to determine the acute oral toxicity of glyphosate acid to Mallard duck (*Anas platyrhynchos*). As no mortalities were observed in a range finder study at a maximum limit dose of 2000 mg a.s./kg bw, this dose was tested for the definitive study. Twenty animals were randomly allocated to two groups, one treatment item group and one control, each comprising five males and five females. On Day 0, a single oral dose was administered by direct intubationof 2000 mg a.s./kg bw to the treatment item group. The control group was treated with vehicle only (0.5 % w/w CMC solution).

Birds were observed for clinical signs of toxicity, behaviour, body weight effects, food consumption and mortality for 14 days after dosing. Body weights were measured individually at test initiation (day 0), and on day 5, 11 and 14 after test initiation. Food consumption for each cage of animals was measured per time interval, covering days 0-7, and days 7-14.

No mortalities and no post-dosing signs of toxicity were observed. Furthermore, the body weight was not affected by the treatment. There were no treatment-related effects on food consumption and no abnormalities were detected at necropsy of the animals 14 days after treatment.

All validity criteria according to the OECD guideline 223 were fulfilled.

The acute oral LD_{50} for Mallard duck exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL was determined to be 2000 mg a.s./kg bw.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White / off-white crystalline powder
Lot/Batch #:	H95 D161A
Purity:	95.3% w/w

2. Vehicle and/or positive control: 0.5% carboxymethyl cellulose (CMC) (vehicle)

3. Test organisms:

Species:	Mallard duck (*Anas platyrhynchos*)
Age:	Young adults, approx. 23 weeks old

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Sex	Males and females
Weight	903 - 1114g (at test initiation)
Source:	[redacted] UK
Loading	Approx. 4.5 m2 for 5 birds
Diet/Food:	Proprietary avian food, *ad libitum*
Acclimation period:	5 weeks prior to dosing
Fasting	16 to 17 hours prior to dosing

4. Environmental conditions:

Temperature:	15 – 22°C
Humidity:	42 – 74%
Photoperiod:	14 hours light / 10 hours dark

B. STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range-finding study, an acute oral toxicity test was performed as a limit test by administering a single limit dose of 2000 mg a.s./kg bw. (dissolved in 0.5% carboxymethyl cellulose) by direct intubation to ten juvenile Mallard ducks (5 males and 5 females)in one treatment group. In addition, one control group comprising 5 males and 5 females was administered an equivalent volume of the vehicle (CMC) only, at a dose rate of 2 mL/kg bw. After dosing, birds where fed *ad libitum* throughout the study.

2. Observations: Birds were caged and observed for signs of toxicity, abnormal behaviour and mortality continuously for one hour after dosing, then at intervals throughout day 0 and twice daily thereafter. Food consumption was measured per time interval, covering day 0-7, and day 7-14.Each animal was weighed at least on day 0, 5, 11 and 14.On day 14, all surviving animals were sacrificed and a gross macroscopic examination was carried out. The necropsy comprised a general inspection of major visceral organs.

3. Statistical calculations: Since no mortality was reported, no statistical calculation of LD_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

The $LD_{50}$and NOEL values are given below based on nominal doses

Endpoints	Glyphosate acid [mg a.s/kg bw]
LD_{50}	> 2000
NOEL	2000

Table 8.1.1-4: Effects of glyphosate acid on body weight and food consumption of Mallard duck

Glyphosate acid [mg a.s./kg bw]			Control	2000
Average body weight per animal [g] (± SD)				
Body weight	Day 0	male	1011 ± 41.5	1012 ± 76.4
		female	1072 ± 128.4	1018 ± 81.1
	Day 5	male	1101 ± 33.5	1048 ± 49.6
		female	1170 ± 160.0	1082 ± 60.8

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	Day 11	male	1096 ± 54.8	1052 ± 69.6
		female	1191 ± 155.0	1175 ± 41.4
	Day 14	male	1104 ± 51.8	1053 ± 65.9
		female	1171 ± 122.6	1156 ± 66.5
Body weight change	Days 0-14	male	93 ± 30.9	42 ± 12.2
		female	99 ± 86.0	138 ± 110.8
Mean food consumption per animal [g/bird/day]				
Food consumption	Day 0-7	male	79	80
		female	131	121
	Day 7-14	male	72	76
		female	130	138

B. OBSERVATIONS

No mortalities and no post-dosing signs of toxicity were observed in any treatment and all animals remained in good health throughout the study. Furthermore, the body weight was not affected adversely by the treatment. There were equally no treatment-related effects on food consumption and no abnormalities were detected at necropsy of the animals 14 days after treatment.

All validity criteria according to OECD 223 were fulfilled, as no non-incidental death was observed in the control groups.

III. CONCLUSION

The acute oral LD_{50} for Mallard duck exposed to glyphosate acid was determined to be > 2000 mg a.s./kg bw. The NOEL was determined to be 2000 mg a.s./kg bw.

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Annex point	Author(s)	Year	Study title
IIA 8.1.1./05	[redacted]	2003	MON 78623: AN ACUTE ORAL TOXICITY STUDY WITH THE NORTHERN BOBWHITE [redacted] Report No: [redacted]-2002-151 Date: 2003-10-10 GLP: no Not published

Guideline: OPPTS 850.2100
FIFRA subdivision E, Section 71-1

Deviations to OECD 223: None.

Dates of experimental work: 2002-10-15 to 2002-10-29

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Executive Summary

A laboratory study was performed to determine the acute oral toxicity of glyphosate K-salt (MON 78623) to bobwhite quail (*Colinus virginianus*). Ten quails (5 male, 5 female) per dose rate received nominal dietary doses of 291, 484, 807, 1344 and 2241 mg glyphosate acid equivalent/kg bw by oral gavage. The control group was administered an equivalent volume of the diluent (deionised water).

Birds were individually observed for mortality, clinical signs of toxicity and abnormal behaviour twice daily for 8 days after study initiation. Body weights were measured at study initiation and after 3, 7 and 14 d. Food consumption for each cage of animals was measured per time interval covering day 0 – 3, 4 – 7 and 8 – 14.

No mortalities were observed at any dose tested and in control treatments. A number of birds showed a ruffled appearance at doses of 484 and higher. At 1344 and 2241 mg glyphosate acid equivalent/kg bw some birds were lethargic. A treatment related loss of body weight was observed at 2241 mg glyphosate acid equivalent/kg bw, while no effects on feed consumption were noted.

All validity criteria according to the current guideline OECD 223 were fulfilled.

The acute LD_{50} for Northern bobwhite exposed to glyphosate K-salt was determined to be > 2241 mg glyphosate acid equivalent/kg bw (nominal). The NOEC was determined to be 484 mg glyphosate acid equivalent/kg bw (nominal).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 78623
Description: Yellow liquid
Lot/Batch #: GLP-0108-11688-F
Purity: 47.7% glyphosate acid

2. Vehicle and/or positive control: Deionised water

3. Test organisms:

Species: Northern bobwhite, Bobwhite quail (*Colinus virginianus*)
Age: Young adults, 30 weeks

Sex	5 male, 5 female per treatment/ control group
Weight	176 - 248 g (at test initiation)
Source:	[redacted] USA
Diet/Food:	Game bird ration, *ad libitum* during acclimation and during the test, 18 h fasting prior to test start. Birds were given water soluble antibiotic in their drinking water for seven days after arrival in the laboratory.
Acclimation period:	Approx. 4 months
Fasting	18 hours prior to dosing

4. Environmental conditions:

Temperature:	22.0 ± 0.2 °C
Relative humidity:	43% ± 8%
Photoperiod:	8 h light, 16 h dark

B. STUDY DESIGN AND METHODS

1. Experimental treatments: In an acute oral toxicity test, bobwhite quail were given nominal doses of 291, 484, 807, 1344 and 2241mg glyphosate acid equivalent/kg bw by oral gavage and observed the following 14 d for mortality, clinical signs of toxicity, abnormal behaviour, body weight change and feed consumption. Ten quails (5 male, 5 female) were assessed per dose and control group. The control group was given diluent only.

2. Observations: After test initiation, birds were observed twice daily for mortality, clinical signs of toxicity and abnormal behaviour. Body weights were measured at study initiation and after 3, 7 and 14 d. Average feed consumption was determined by pen for each group for day 0 – 3, 4 – 7 and 8 – 14, by measuring the weight change of the presented feed.

3. Statistical calculations: Since the mortality was < 50%, no statistical calculation of LC_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

The LD_{50} and NOEL values are given below based on nominal doses

Endpoints	mg glyphosate acid equivalent/kg bw
LD_{50}	> 2241
NOEL	484

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Table 8.1.1-5 Effects of glyphosate K-salt on body weight, food consumption of Northern bobwhite quail

Glyphosate acid equivalent [mg/kg bw]	Control	291	484	807	1344	2241
Mortality						
Day 14	0	0	0	0	0	0
Clinical signs						
Ruffled appearance	0	0	2[1]	4	5	2
Lethargy	0	0	0	0	1	1
Mean body weight [g] (male/female)						
Day 0	224/197	221/208	222/207	219/206	219/221	225/212
Day 14	221/201	224/209	223/210	221/209	221/226	223/216
Feed consumption [g] (male/female)						
Day 0 - 3	31/15	28/21	27/26	18/23	21/15	17/23
Day 4 - 7	28/21	29/22	23/26	20/24	23/23	25/28
Day 8 - 14	25/16	24/17	19/20	21/20	20/18	17/18

[1] Not considered to be treatment related due to the timing and isolated nature of the signs noted.

B. OBSERVATIONS

There was no treatment-related mortality observed. One control male suffered a leg injury during body weight procedures and lost weight afterwards.

Numerous birds developed foot injuries during the study, which were not treatment related. At 2241 mg glyphosate acid equivalent/kg bw one male received a foot injury. One male and one female in the 484 mg glyphosate acid equivalent/kg bw group got foot lesions with associated lameness and/or ruffled appearance. This was considered to be incidental to the treatment. At 807 1344 and 2241 mg glyphosate acid equivalent/kg bw a number of birds showed a ruffled appearance. At the two highest test concentrations also lethargy was observed. No dose-response related increase of toxicity signs was noted.

When compared to the control group, no treatment related effects on body weight were noted except for the highest test concentration of 2241 mg glyphosate acid equivalent/kg bw. No treatment related effect on feed consumption was observed.

All validity criteria according to OECD 223 were fulfilled, as no non-incidental death was observed in the control groups.

III. CONCLUSION

The acute LD_{50} for northern bobwhite exposed to glyphosate K-salt was determined to be > 2241 mg glyphosate acid equivalent/kg bw (nominal). The NOEC was determined to be 484 mg glyphosate acid equivalent/kg bw (nominal).

IIA 8.1.2 Avian dietary toxicity (5-day) test in a quail species or in a mallard duck

Under the revised 91/414 guidance as well as the current guidance document on Risk Assessment for Birds and Mammals (EFSA 2009) short term toxicity data for birds are not required.

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IIA 8.1.3 Avian dietary toxicity (5-day) test in a second unrelated species

Please refer to IIA 8.1.2

IIA 8.1.4 Subchronic and reproductive toxicity to birds

Annex point	Author(s)	Year	Study title
IIA 8.1.4./01	[redacted]	1999	Glyphosate Acid: A Reproduction Study with the Northern Bobwhite (*Colinus virginianus*) [redacted] Report No: 123-186 Date: 1999-05-13 GLP: yes Not published

Guideline: FIFRA Guideline 71-4
OECD Guideline 206

Deviations to OECD 206: None

Dates of experimental work: 1998-05-29 to 1998-11-23

Executive Summary

In a reproductive toxicity study, glyphosate acid was fed for 20 weeks to young adult bobwhite quail (*Colinus virginianus*). Thirty-two adult quail (1 male and 1 female per pen and 16 pens per study group) per dosage and control received nominal dietary doses of 500, 1000 and 2250 mg glyphosate acid/kg feed. Birds were allowed to lay eggs for approximately 10 weeks. Eggs were collected, incubated and allowed to hatch. During egg deposition period, incubation and post hatching period, eggs and hatchlings were observed for different reproductive parameters, encompassing total egg production, number of eggs cracked, eggshell thickness, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 days-old hatchlings and 14 day survivorship.

Results showed no treatment-related mortalities, overt symptoms of toxicity, or treatment effects upon body weight or feed consumption at any of the dietary doses tested. In addition, no treatment-related effects upon any of the reproductive parameters measured at any of the test doses were observed.

All validity criteria according to the OECD guideline 206 were fulfilled.

Based on the results of this study, the NOEL for bobwhite quail exposed to glyphosate acid in a reproduction study was determined to be 2250 mg glyphosate acid/kg feed.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: P24
Purity: 95.6%

2. Vehicle and/or positive control: None

3. Test organisms:

Species: Bobwhite quail (*Colinus virginianus*)
Age: Young adults, 30 weeks (at test initiation)

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Sex	Males and females
Weight	196 to 250 g (at test initiation)
Source:	[redacted] USA
Loading	Approx. 0.138 m^2 for 2 birds (1 males and 1 female per pen)
Feed/Diet:	Game bird ration, *ad libitum*
Acclimation period:	10 weeks

4. Environmental conditions:

Temperature:	23.1 ± 1.8°C (adults); 27.3± 1.2°C (hatchling) 38°C (brooding compartment)
Humidity:	66 ± 12% (adults); 40 ± 17% (hatchling)
Photoperiod:	17 hours light / 7 hours dark, (approx. 265 lux)

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A reproductive toxicity study was performed by feeding adult bobwhite quail *ad libitum* on a series of 3 nominal dietary doses, encompassing 500, 1000 and 2250 mg /kg feed. Sixteen replicates (1 male and 1 female per pen) were used for each treatment group and control. The birds were exposed to the treated diets for approximately 20 weeks and were evaluated for treatment-related effects upon bird health and reproduction. Eggs were collected daily and stored at 13.6 ± 0.6°C and 82 ± 8% relative humidity. All eggs laid within a week were considered as one lot and incubated in a Petersime Incubator. On day 21 of incubation, eggs were placed in a Petersime Hatcher and allowed to hatch. The hatchlings were maintained on untreated diet until 14 days of age. Homogeneity of the test substance in treated diets was evaluated by collecting 6 samples of each treatment group on day 0 of week 1. During weeks 2, 3, 4, 8, 12, 16 and 20 of the test, a single sample was collected from the control diet and an additional duplicate sample was collected from treatment group diet, to measure and/ or verify test concentrations.

2. Observations: Adult birds were observed daily for signs of toxicity and abnormal behaviour throughout the study. Adult body weight was measured at study initiation and termination, in addition to on weeks 2, 4, 6, and 8. For each pen, food consumption was measured weekly throughout the study except for the last interval, where food consumption was measured over a 6 day period. At the end of each week, all collected eggs were counted and a single egg was randomly selected for eggshell thickness measurements. The remaining eggs were candled to detect egg shell cracks or abnormal eggs before incubation. During the incubation period, eggs were candled again on day 11 or 12 to evaluate embryo viability and on day 21 to determine embryo survival. During the study, total egg production, number of eggs cracked, eggshell thickness, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 day old hatchlings and survivorship of 14 day old hatchlings were determined.

3. Statistical calculations: An analysis of variance (ANOVA) was used to determine significant differences among the groups followed by Dunnett's multiple comparison procedure as the post-hoc test.

II.RESULTS AND DISCUSSION

A. FINDINGS

The NOEL value is given below based on nominal doses:

Endpoints	glyphosate acid [mg a.s./kg feed]
NOEL reproduction	2250

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Table 8.1.4-1: Effects of glyphosate acid on reproductive performance of bobwhite quail over 10 weeks.

Glyphosate acid [mg a.s./kg feed]	Control	500	1000	2250
Reproductive performance				
Number of eggs laid per female [mean]	47.7	42.2	39.0	44.0
Eggs laid/maximum laid [%]	70	62	57	65
Eggs cracked/egg laid [%]	5	4	11	5
Viable embryos/egg set [%]	81	91	94	92
Live 3-week embryos/viable embryos [%]	99	98	98	98
Hatchlings/live 3-week embryos [%]	95	95	95	99
14-day-old survivors/hatchlings [%]	93	96	95	96
Hatchlings/egg set [%]	75	85	88	88
14-day-old survivors/egg set [%]	70	82	83	85
Hatchlings/maximum set [%]	50	51	44	54
14-day-old survivors/ maximum set [%]	46	49	43	52
Eggshell thickness				
Mean shell thickness [mm]	0.220	0.228	0.222	0.216
Body weight of hatchling				
Mean body weight [g]	6	6	7	6
Body weight of 14-day old survivors				
Mean body weight [g]	26	28	28	27

Table 8.1.4-2: Effects of glyphosate acid on adult bodyweight and feed consumption of adult bobwhite quail.

Glyphosate acid [mg a.s./kg feed]		Control	500	1000	2250
Average body weight [g]					
Test initiation	M	215	223	216	214
	F	219	219	216	218
Test termination	M	219	229	219	215
	F	250	248	238	239
Body weight change (test start - test end)	M	4	6	3	2
	F	31	29	21	23
Average feed consumption [g/bird/day]					
Week 1	M + F	12	12	12	12
Week 5	M + F	12	12	12	13
Week 10	M + F	19	18	19	20
Week 15	M + F	26	26	26	28
Week 20	M + F	25	26	25	26

M male
F female

B. OBSERVATIONS

No treatment-related mortality of parental birds exposed to glyphosate acid was observed. No overt symptoms of toxicity or treatment related effects upon body weight or feed consumption were observed at any dietary dose tested. In addition, no treatment-related effects of reproductive parameters were observed at any dose tested.

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Analysis of samples resulted in measured concentrations of 100%, 99% and 96% of the nominal test doses of 500, 1000 and 2250 mg glyphosate acid/kg feed, respectively.

All validity criteria according to OECD 206 were fulfilled, as the mortality of the control did not exceed 10% at the end of the test and the average number of 14-day-old survivors per hen in the control was greater than 12. Also, the average egg shell thickness for the control group was greater than 0.19 and the lowest treatment level did not result in compound-related mortality or observable toxic effects.

III.CONCLUSION

The NOEL for bobwhite quail exposed to glyphosate acid in a reproduction study was determined to be 2250mg glyphosate acid/kg feed.

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Annex point	Author(s)	Year	Study title
IIA 8.1.4./02		1999	Glyphosate Acid: A reproduction Study with the Mallard (*Anas platyrhynchos*) Report No: 123-187 Date: 1999-01-11 GLP: yes Not published

Guideline: FIFRA Guideline 71-4
OECD Guideline 206

Deviations to OECD 206: None

Dates of experimental work: 1998-05-29 to 1998-12-03

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Executive Summary

In a reproductive toxicity study, glyphosate acid was fed to mallard ducks (*Anas platyrhynchos*) for a total duration of 21 weeks. Thirty-two adult mallards (1 male and 1 female per pen and 16 pens per test dose and control) per dosage and control received nominal dietary doses of 500, 1000 and 2250 mg glyphosate acid/kg feed.

Birds fed on the treated diet were allowed to lay eggs for approximately 11 weeks. Eggs were collected, washed and incubated and allowed to hatch. During egg deposition period, incubation and post hatching period, eggs and hatchlings were observed for different reproductive parameters, encompassing the total egg production, number of eggs cracked, eggshell thickness, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 day old hatchlings and survivorship of 14 day-old hatchlings.

Results showed no treatment-related mortalities, overt symptoms of toxicity, or treatment effects upon body weight or feed consumption at any of the dietary doses tested. In addition, no treatment-related effects upon any of the reproductive parameters measured at any of the test doses were observed. All validity criteria according to the OECD guideline 206 were fulfilled.

Based on the results of this study, the NOEL for mallard ducks exposed to glyphosate acid in a reproduction study was determined to be 2250mg glyphosate /kg feed, which was the maximum dose tested.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: P24
Purity: 95.6%

2. Vehicle and/or positive control: None

3. Test organisms:

Species: Mallard duck (*Anas platyrhynchos*)
Age: 21 weeks (at test initiation)

Sex:	Males and females
Weight:	868 to 1259 g (at test initiation)
Source:	USA
Loading:	Approx. 0.675 m2 for 2 birds (1 males and 1 female per pen)
Feed/Diet:	Game bird ration, ad libitum
Acclimation period:	6 weeks

4. Environmental conditions:

Temperature:	22.4 ± 0.9°C (adults); 29 °C (hatchling); 38°C (brooding compartment)
Humidity:	69 ± 13% (adults); 61 ± 15% (hatchling)
Photoperiod:	17 hours light / 7 hours dark, (approx. 292 Lux)

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A reproductive toxicity study was performed by feeding young adult mallard ducks *ad libitum* on a series of 3 nominal dietary doses, encompassing 500, 1000, and 2250 mg glyphosate acid/kg feed. Sixteen replicates (1 male and 1 female per pen, 16 pen per treatment group) were used for each treatment group and control. The birds were exposed to the treated diets for approximately 21 weeks, and were evaluated for treatment-related effects on bird health and reproduction. Eggs were collected daily, washed and stored in a cold room at 13.6 ± 0.6°C and 82 ± 8% relative humidity. All eggs laid within a week were considered as one lot and were incubated in a Petersime incubator. On day 24 of incubation, eggs were placed in a Petersime hatcher and were allowed to hatch. The hatchlings were maintained on untreated diet until 14 days of age. Homogeneity of the test substance in treated diet was evaluated by collecting 6 samples from each treatment group on day 0 of week 1. During weeks 2, 3, 4, 8, 12, 16 and 20 of the test, a single sample was collected from the control diet and an additional duplicate sample was collected from treatment group diet, to measure and/ or verify test concentrations.

2. Observations: Parental birds were observed daily throughout the study for signs of toxicity and abnormal behaviour. Adult body weights were measured at study initiation and termination in addition to on weeks 2, 4, 6, and 8 of the adult in-life period. For each pen, feed consumption was measured weekly. At the end of each week, all eggs collected were counted and selected by indiscriminate draw for eggshell thickness measurement. The remaining eggs were candled to detect egg shell cracks or abnormal eggs before incubation. During the incubation period, eggs were candled again on day 14 to investigate embryo viability and on day 21 to determine embryo survival.

During the study, total egg production, number of eggs cracked, eggshell thickness, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 day old hatchlings and survivorship of hatchlings after 14 days were determined.

3. Statistical calculations: An analysis of variance (ANOVA) was used to determine significant differences among the groups and Dunnett's multiple comparison procedure was used as post-hoc test.

II. RESULTS AND DISCUSSION

A. FINDINGS

The NOEL value is given below based on nominal doses.

Endpoints	glyphosate acid [mg/kg feed]
NOEL reproduction	2250

Table 8.1.4-3: Effects of glyphosate acid on reproductive performance of mallard duck[1]

Glyphosate acid [mg/kg feed]	Control	500	1000	2250
Reproductive performance				
Number of eggs laid per female [mean]	43.6	40.1	40.2	44.3
Eggs laid/maximum laid [%]	61	56	56	62
Eggs cracked/eggs laid [%]	2	1	1	2
Viable embryo/egg set [%]	73	68	93	81
Live 3-week embryos/viable embryos [%]	98	99	99	99
Hatchlings/live 3-week embryos [%]	91	89	84	88
14-day-old survivors/hatchlings [%]	100	91	98	99
Hatchlings/egg set [%]	66	60	78	72
14-day-old survivors/egg set [%]	65	58	76	71
Hatchlings/maximum set [%]	34	31	43	42
14-day-old survivors/ maximum set [%]	34	30	42	42
Eggshell thickness				
Number of eggs measured	58	59	61	65
Mean shell thickness [mm]	0.388	0.374	0.373	0.376
Body weight of hatchling				
Number of juvenile quails weighted	329	302	414	440
Mean body weight [g]	36	34	35	34

[1] values represent pen means for experimental groups

Table 8.1.4-4: Effects of glyphosate acid on adult bodyweight and feed consumption of adult mallard duck.

Glyphosate acid [mg/kg feed]		Control	500	1000	2250
Average body weight [g]					
Test initiation	male	1091	1103	1106	1107
	female	1024	1021	1019	999
Test termination	male	1161	1105	1134	1088
	female	1114	1104	1112	1080
Body weight change (test start - test end)	male	68	0	27	-19
	female	99	76	90	81
Average feed consumption [g/bird/day]					
Week 1		89	102	86	93
Week 5		95	93	92	101
Week 10		137	125	117	127
Week 15		193	193	168	198
Week 21		169	167	170	173

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B. OBSERVATIONS

No treatment-related mortality of parental birds exposed to glyphosate acid was observed. Also, no overt symptoms of toxicity or treatment related effects on body weight or feed consumption were observed at any dose tested. In addition, no treatment-related effects on any of the reproductive parameters were observed.

Analyses of samples of diet resulted in concentrations of 100%, 99% and 96% of the nominal test doses of 500, 1000 and 2250 mg glyphosate acid/kg feed, respectively.

All validity criteria according to OECD 206 were fulfilled, as the mortality of the control group did not exceed 10 % at the end of the test and the average number of 14-day-old survivors per hen in the control was greater than 14. Also, the average egg shell thickness for the control group was greater than 0.34 and the lowest treatment level did not result in compound-related mortality or observable toxic effects.

III. CONCLUSION

The NOEL for mallard ducks exposed to glyphosate acid in a reproduction study was determined to be 2250mg glyphosate acid/kg feed.

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Annex point	Author(s)	Year	Study title
IIA 8.1.4./03	[redacted]	1978	One-Generation Reproduction Study – Bobwhite Quail; Glyphosate Technical. [redacted] Report No: 139-141 Date: 1978-11-01 GLP: no Not published

Guideline: Not stated

Deviations: none

Dates of experimental work: 1978-03-01 to 1978-08-01

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Executive Summary

In a 17 week reproductive toxicity study, glyphosate acid was fed to bobwhite quail (*Colinus virginianus*). Three adult quail per pen (1 male and 2 female) in 12 replicates per treatment received three nominal dietary doses of 50, 200 and 1000 mg glyphosate acid/kg diet.

Birds were fed on the treated diet for 9 weeks prior to egg deposition and were allowed to lay eggs for 8 weeks. Eggs were collected, incubated and allowed to hatch. During the egg deposition period, incubation and post hatching period, eggs and hatchlings were observed for different parameters encompassing total egg production, number of eggs cracked, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 day-old hatchlings, 14 day survivorship, egg weight and eggshell thickness.

Results showed significant reduction in egg weight occurring at the highest test item dose of 1000 mg glyphosate acid/kg diet. However, no further effects on reproduction were observed at this dose level. Therefore, the reduction in egg weight was not considered to be biologically relevant. A high incidence of eggshell cracks was noted during the course of this reproduction study, which can be attributed to the fact that the specimens were inadvertently not debeaked prior to study initiation.

Based on the results of this study, the NOEL for the bobwhite quail exposed to glyphosate acid in a one-generation reproduction study was determined to be 1000 mg glyphosate acid/kg feed.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder with a slight odour
Lot/Batch #: XHI 162
Purity: 83% (measured)

2. Vehicle and/or positive control: Corn oil (vehicle)

3. Test organisms:

Species: Bobwhite quail (*Colinus virginianus*)
Age: 5 months old (young adults)
Sex Males and females
Weight Not stated
Source: [redacted]
Loading 1 males and 2 females per pen

Diet/Diet: Game bird breeder ration, *ad libitum*

Acclimation period: Not stated

4. Environmental conditions:

Temperature: 21.1 – 26.7°C (research facility)
15.6°C (eggs storage), 37.4 - 37.6°C (eggs incubation)

Humidity: 55% (eggs storage)

Photoperiod: 9 hours light / 15 hours dark (first 6 weeks)
17 hours light / 7 hours dark (following 16 weeks)

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A reproductive toxicity study was performed by feeding three adult Bobwhite quails(1 male and 2 females per pen) per replicate *ad libitum* on a series of 3 nominal dietary doses, encompassing 50, 200 and 1000 mg glyphosate acid/kg diet. The diet was prepared by incorporating appropriate concentrations of the test item and corn oil into the aliquots of basal diet. Twelve replicates were exposed per treatment group and control. The birds were exposed for nine weeks to the treated diet prior to egg deposition and for additional eight weeks during egg collection. Eggs were collected daily, stored at 15.6°C and 55% relative humidity and were cleaned weekly. The eggs were then incubated at 37.5 ± 0.06°C. On day 19 of incubation, the eggs were placed in a Humidaire hatcher and allowed to hatch. All hatchlings were housed according to the appropriate parental grouping and maintained on control diet until 14 days of age.

2. Observations: Body weights were recorded at study initiation, 5 weeks after study initiation prior to onset of egg deposition and at termination of the study. Food consumption was recorded every second week throughout the study. All eggs were candled on day 0 of incubation for eggshell cracks, on day 14 to measure embryo viability, and on day 19 to measure embryo survival. Weekly throughout the egg deposition period, one egg of each pen in each group was randomly selected for egg weight and eggshell thickness measurement. During the study total egg production, number of eggs cracked, egg set, embryo viability, embryo survival, number of hatchlings, body weight of new hatchlings, body weight of 14 days-old hatchlings, 14 day survival, egg weight and eggshell thickness were determined.

3. Statistical calculations: To evaluate differences between reproductive parameters, Student's t-test was used.

II. RESULTS AND DISCUSSION

A. FINDINGS

The NOEL value is given below based on nominal doses:

Endpoints	glyphosate acid [mg a.s./kg feed]
NOEL reproduction	1000

Table 8.1.4-5: Effects of glyphosate on reproductive parameters of bobwhite quail

Glyphosate acid [mg test/kg diet]	Control	50	200	1000
Reproductive success				
Number of eggs laid per hen in 8 weeks (mean)	31.9	28.0	28.0	32.5
Number of eggs cracked [%]	9.7	7.6	9.2	6.3
Viable embryos of egg set	85.9	74.6	85.1	81.7
Live 3-week embryos of viable embryos [%]	97.3	97.2	97.5	96.5
Hatchlings of live 3-week embryos [%]	81.5	70.3	73.4	74.4

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14-day-old survivors of normal hatchlings [%]	95.5	93.1	95.7	93.5
14- day-old survivors per hen [a]	18.7	12.3	14.8	16.7
Egg weight				
Mean egg weight [g]	10.3	9.9	10.2	9.4 *
Eggshell thickness				
Mean eggshell thickness [mm]	0.214	0.204	0.211	0.224
Body weight of representative hatchling				
Mean body weight [g]	6.8	6.9	6.9	6.7
Body weight of representative 14-day old survivors				
Mean body weight [g]	22.0	22.2	22.6	22.0

[a]based on 24 hens
* Statistically significant compared to control (Student's t-test)

B. OBSERVATIONS

There were no statistically significant impacts on any reproductive parameters with one exception. A statistically significant reduction in egg weight occurred at the highest test dose of 1000 mg glyphosate acid/kg diet. Although there was a small reduction in egg weight at 1000 mg/kg feed there was not a significant impact on the biologically relevant endpoints that included initial hatchling body weight, 14 day hatchling body weight, egg shell thickness and hatchling survival. Egg weight is not a standard endpoint in guideline avian reproduction studies, it is not included in OECD 206, and was a carryover from poultry performance studies. A high incidence of eggshell cracks was noted during the course of the study. This can be attributed to the fact that the bobwhite quail utilized for this study were inadvertently not debeaked prior to study initiation. In fact, caged quail have a natural propensity to peck at their eggs causing cracks. All current validity criteria were fulfilled, as the mortality of the control did not exceed 10 % at the end of the test and the average number of 14-day-old survivors per hen in the control was≥ 14. Also, the average egg shell thickness for the control group was ≥ 0.34 mm and the lowest treatment level did not result in compound-related mortality or observable toxic effects.

III. CONCLUSION

Based on the overall results of this study, the NOEL for bobwhite quail exposed to glyphosate acid in a one-generation reproduction study was determined to be 1000 mg glyphosate acid/kg diet.

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Annex point	Author(s)	Year	Study title
IIA 8.1.4./04	[redacted]	1978	One-Generation Reproduction Study - Mallard Duck; Glyphosate technical. [redacted] Report No: 139-143 Date: 1978-09-25 GLP: no Not published

Guideline: Not stated

Deviations: N/A

Dates of experimental work: 1978-03-01 to 1978-08-01

Executive Summary

In a reproductive toxicity study, glyphosate acid was fed to mallard ducks (*Anas platyrhynchos*) for17 weeks. Five replicates per dose, containing seven adult ducks (2 males and 5 females per pen)each were treated with nominal dietary doses of 50, 200and 1000 mg glyphosate acid/kg diet for nine weeks. Reproductive parameters were measured for a further eight weeks beginning at the onset of egg laying. Eggs were collected, incubated and allowed to hatch. During the egg deposition period, incubation and post hatching period, eggs and hatchlings were observed for different reproductive parameters, encompassing the total egg production, the number of egg cracked, embryos viability, embryos survival, number of hatchlings, body weight of representative new hatchlings, body weight of representative 14 days-old hatchlings, 14 day-old survivorship, egg weight and the eggshell thickness.
No symptoms of toxicity or behavioural abnormalities at any of the dietary doses tested and in control were observed for the entire test duration for the parental birds exposed to glyphosate. In addition, no mortality was observed in control and treatments groups, except at the highest test item concentration, where a single mortality was observed on week 12 after study initiation. This death was however considered incidental and not compound related. The evaluation of reproductive data and statistical analysis of the above mentioned reproductive parameters demonstrated that glyphosate caused no reproductive impairment at the dose levels tested.

Based on the results of this study, the NOEL for the mallard duck exposed to glyphosate acid in a one-generation reproduction study was determined to be 1000 mg glyphosate acid/kg diet.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder with a slight odour
Lot/Batch #: XHI 162
Purity: 83% a.s.

2. Vehicle and/or positive control: Corn oil (vehicle)

3. Test organisms:

Species: Mallard duck (*Anas platyrhynchos*)
Age: 6 months old (adults, at test initiation)
Sex Males and females
Weight 1047 - 1257 g (at test initiation)

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Source: [redacted]

Loading: Approx. 8.2 m^2 for 7 specimens (2 males and 5 females per pen)

Diet/Diet: Game bird breeder ration, *ad libitum*

4. Environmental conditions:

Temperature: 37.4 – 37.6°C (eggs incubation)

Humidity: 55% (eggs storage)

Photoperiod: outdoor (natural daylight/photoperiod)

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A reproductive toxicity study was performed by feeding7 adult mallard ducks (2 males and 5 females per replicate)*ad libitum*, on a series of 3 nominal dietary doses encompassing 50, 200and 1000 mg glyphosate acid/kg diet. The diet was prepared by incorporating appropriate concentrations oft he test item and corn oil into the aliquots of basal diet. Five replicates were used for each treatment group and the control. The birds were exposed to the treated diet for 9 weeks prior to egg deposition and for additional 8 weeks during egg collection. Eggs were collected daily and stored at 15.6°C and 55% relative humidity and were cleaned weekly. The clean eggs were then incubated at 37.5 ± 0.06°C. On day22 or 23 of incubation, the eggs were allowed to hatch. The hatchlings were housed according to the appropriate parental grouping and maintained on control diet until 14 days of age.

2. Observations: Body weights were recorded at study initiation, 5 weeks after study initiation, prior to the onset of egg deposition, and at termination of the study. Food consumption was recorded bi-weekly throughout the study. All eggs were candled on day 0 of incubation for eggshell cracks, on day 14 to measure embryo viability and to remove any *E coli*-contaminated eggs, and on day 21 to measure embryo survival. Weekly throughout egg deposition period, one egg from each pen in each experimental group and the controls was randomly selected for egg weight and eggshell thickness measurement. During the study, the total egg production, the number of eggs cracked, embryos viability, embryos survival, number of hatchlings, body weight of representative new hatchling, body weight of representative 14 days-old hatchlings, 14 day-old survivorship, egg weight and the eggshell thickness were determined.

3. Statistical calculations: To evaluate the differences between each of the above-mentioned reproductive parameters, Student's t-test was used.

II. RESULTS AND DISCUSSION

A. FINDINGS

The NOEL value is given below based on nominal doses:

Endpoints	glyphosate acid [mg a.s./kg feed]
NOEL reproduction	1000

Table 8.1.4-6: Effects of glyphosate on reproductive parameters of Mallard duck

Glyphosate technical [mg test/kg diet]	Control	50	200	1000
Reproductive success				
Number of eggs laid per hen in 8 weeks	28	23	28	29
Number of eggs cracked [%]	3	5	5	6
Viable embryos of egg set	90	93	85	86
Live 3-week embryos of viable embryos [%]	96	93	95	95

Glyphosate technical [mg test/kg diet]	Control	50	200	1000
Hatchlings of live 3-week embryos [%]	74	77	77	81
14-day-old survivors of normal hatchlings [%]	97	99	98	96
14- day-old survivors per hen [a]	16	14	15	16
Egg weight				
Number of eggs analysed	38	38	38	39
Mean egg weight[g]	57.5	58.3	56.3	58.9
Eggshell thickness				
Number of eggs analysed	38	38	38	39
Mean shell thickness [mm]	0.394	0.375	0.372	0.375
Body weight of representative hatchling				
Number of ducklings analysed	72	73	72	73
Mean body weight[g]	33	33	32	34
Body weight of representative 14-day old survivors				
Number of ducklings analysed	72	72	72	73
Mean body weight[g]	217	206	208	205

[a] based on 25 hens

B. OBSERVATIONS

For the parental birds exposed to glyphosate, there no symptoms of toxicity or behavioural abnormalities were recorded at any of the dietary doses tested of the control treatments for the entire test duration. In addition, no mortality was observed in control and treatments groups, except for the highest test dose, at which a single mortality was observed on week 12 after study initiation. This death was however considered incidental, and not compound related. The evaluation of the reproductive data and statistical analysis of above-mentioned reproductive parameters demonstrate that glyphosate caused no reproductive impairment at the dose levels tested. All validity criteria according to current guidelines were fulfilled, as the mortality of the control did not exceed 10 % at the end of the test and the average number of 14-day-old survivors per hen in the control was ≥ 14. Also, the average egg shell thickness for the control group was ≥ 0.34 mm and the lowest treatment level did not result in compound-related mortality or observable toxic effects.

III. CONCLUSION

Based on the results of this study, the NOEL for Mallard duck exposed to glyphosate acid in a one-generation reproduction study was determined to be 1000 mg glyphosate acid/kg diet.

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IIA 8.2 Fish toxicity

As summarized in the 2001 EU evaluation of glyphosate, glyphosate acid has low acute and chronic toxicity to aquatic invertebrates and vertebrates. The chronic NOEC for the 21-day *Daphnia* life-cycle study and the 255 day fish full life-cycle study were 30 mg a.s./L and 25.7 mg a.s./L, respectively. The lowest endpoint was for an algal species, but these endpoints still provided a TER >10, and after a recent evaluation of these studies with *Skeletonema* they is no longer considered valid studies. Additionally, the toxicity data show that the IPA-sa1t and AMPA are less toxic than glyphosate acid. New studies not included in the 2001 EU evaluation of glyphosate, for the aquatic metabolites AMPA and HMPA confirm the low toxicity to aquatic animals.

Bioconcentration studies examining uptake and elimination in bluegill, crayfish and molluscs have been performed for glyphosate and were reviewed in the 2001 EU evaluation of glyphosate. It was concluded in the evaluation that the potential for glyphosate to bioconcentrate is considered low. Because glyphosate does not bioconcentrate and has low chronic toxicity to aquatic invertebrates, it was further concluded in the 2001 EU evaluation that a study with a sediment dwelling organism is not required. The 2001 EU evaluation of glyphosate also concluded because AMPA has low toxicity an AMPA study with a sediment dwelling organism is not required.

The aquatic data presented below were generated in accordance with OECD or equivalent guidelines. Only data from tests considered valid or studies considered in the 2001 EU evaluation of glyphosate are listed in the tables below. Further only Tier II summaries are presented for those studies that were not reviewed during the 2001 EU evaluation and that meet current quality, performance and guideline criteria.

IIA 8.2.1 Acute toxicity of the active substance to fish

A summary of all available relevant and compliant (including data already reviewed during the 2001 EU evaluation for glyphosate), glyphosate salts and AMPA are presented for trout and warm water species in Table 8.2.1-1. This section follows the OECD format in terms of content but does not include sub-bullets 8.2.1.1 (rainbow trout), 8.2.1.2 (warm water fish species) and 8.2.1.3 (acute toxicity of metabolites and degradation products).

Table 8.2.1-1: Acute toxicity of glyphosate acid, salts of glyphosate and AMPA to fish

Species	Test design	LC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/ GLP	2001 EU evaluation monograph reference
		Glyphosate acid			
Oncorhynchus mykiss	96 h static	71.4	34.9	-78-165 1978/no	95-00014
Oncorhynchus mykiss	96 h static	95 - 171	95	271631 1990/yes	95-00011
Oncorhynchus mykiss	96 h static	130	32	IIA 8.2.1/01 5552/B 1995/yes	NA
Oncorhynchus mykiss	96 h	>100	>100		95-00536 1995
Oncorhynchus mykiss	96 h	**38**	10		95-00016 1972/no
Lepomis macrochirus	96 h static	120	-	-78-1123 ,	95-00013

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Species	Test design	LC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/ GLP	2001 EU evaluation monograph reference
				1978/no	
Lepomis macrochirus	96 h static	47	32	IIA 8.2.1/02 5553/B 1995/yes	NA
Lepomis macrochirus	96 h static	133.3 - 200	133.3	271642 1991/yes	95-00012
Lepomis macrochirus	96 h	78	32		95-00016 1978/no
Danio rerio	96 h semi-static	122.91	56	IIA 8.2.1/03 -D61.47/99 2000/yes	NA
Cyprinus carpio	96 h semi-static	>100	100	IIA 8.2.1/04 2060/015 2006/yes	NA
Cyprinus carpio	96 h	115			95-00015 , 1990
Glyphosate-IPA salt					
Oncorhynchus mykiss	96 h static	2192 1624 mg a.e./L	517 383 mg a.e./L	80-91-2328-03-93 1993/yes	94-00157
Oncorhynchus mykiss	96 h	**>1000** 741 mg a.e./L	1000 741 mg a.e./L		94-01161 , 1981
Leuciscus idus	96 h static	>5000 3704 mg a.e./L	5000 3704 mg a.e./L	80-91-2328-02-93 1993/yes	94-00156
Lepomis macrochirus	96 h	**>1000** 741 mg a.e./L	560 415 mg a.e./L		95-00712 , 1981
Glyphosate K-salt					
Oncorhynchus mykiss	96 h static	1227	157	IIA 8.2.1/07 -2002-149 2003/yes	NA
AMPA					
Oncorhynchus mykiss	96 h static	>100	100	IIA 8.2.1/05 232469 1998/yes	NA
Oncorhynchus mykiss	96 h static	**520**	32	-90-402 1991/yes	94-01162
Oncorhynchus mykiss	96 h static	180	18	IIA 8.2.1/06 5070/B 1991/yes	NA
Oncorhynchus mykiss	96 h	>180	>8		94-00499 , 1994/?

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

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NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

For fish, several acute toxicity studies exist, which were not included in the previous 2001 EU-Evaluation of glyphosate. These studies are summarised below, confirming the existing ecotoxicological profile of glyphosate. Studies considered in the previous evaluation for Annex I, have been re-evaluated against the current relevant test guideline recommendations. (see 2001 EU-Evaluation of glyphosate) Studies that are considered not to meet with current data requirements, such as those without adequate analytical support, have been excluded from the evaluation of toxicity of glyphosate, its formulations or metabolites.

Annex point	Author(s)	Year	Study title
IIA 8.2.1/01	[redacted]	1995	GLYPHOSATE ACID: Acute Toxicity to rainbow trout (*Oncorhynchus mykiss*) [redacted] Report No: [redacted]5552/B Date: 1995-12-8 GLP: yes Not published

Guideline: EPA FIFRA; Subdivision E, 72-1

Deviations to OECD 203: none

Dates of experimental work: 1995-09-11 to 1995-09-15

Executive Summary

The effect of glyphosate acid to rainbow trout (*Oncorhynchus mykiss*) was evaluated in a 96-hour static toxicity test conducted at nominal test concentrations of 32, 56, 100, 180, 320 and 560 mg a.s./L. A negative control (dilution water only) was also prepared. Ten fish were exposed in the control and in each treatment.

All fish were observed at daily intervals for the 96 hour study duration with mortality and sub-lethal signs of toxicity recorded. Dissolved oxygen, pH and temperature were also measured daily in each test vessel. Samples of control and test media were analysed for glyphosate acid at 0 hours (before fish addition) and after 48 and 96 hours.

Glyphosate acid was not detected in the control group. Overall mean measured concentrations of glyphosate acid in test groups ranged from 91 to 100% of nominal concentrations.

Up to a nominal concentration of 100 mg test item/L, no mortality occurred during the 96 h exposure time. From the next higher concentration of 180 mg test item/L all fish died. Sublethal effects like dark discolouration and loss of balance were recorded starting with 56 mg test item/L while a strong correlation between pH value and test item concentrations was observed. At 180 mg test item/L, the pH was reduced to 3.5 and lower. All validity criteria according to the guideline OECD 203 were fulfilled.

The 96 hour LC_{50} value for rainbow trout exposed to glyphosate acid was determined to be 130 mg/L (nominal) with a 95% confidence interval of 100 to 180 mg/L. The 96 hour NOEC value was 32 mg glyphosate acid/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

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Test item: Glyphosate acid
Description: White solid
Lot/Batch #: P24
Purity: 95.6%

2. Vehicle and/or positive control: Filtered and dechlorinated tap water

3. Test organism:

Species: Rainbow trout (*Oncorhynchus mykiss*)
Age: Juvenile
Size: 40 – 71 mm (mean: 57 mm)
Body weight of the animals: 1.16 – 4.56 g/ fish (mean: 2.68 g)
Loading: 0.89 g fish/L (10 fish per 30 litres of test medium)
Source: [redacted] UK
Diet/Food: no feeding for 48 hours prior to test and during the total test period
Acclimation period: 32 days

4. Environmental conditions:

Temperature: 11.5 – 12.6°C
Photoperiod: 16 hours
pH: Control (start – 96 h): 7.7 - 7.0
32 mg/L (start – 96 h): 6.4 – 6.2
56 mg/L (start – 96 h): 5.9 – 6.0
100 mg/L (start – 96 h): 4.7 – 5.1
180 mg/L (start – 24 h): 3.5
320 mg/L (start – 24 h): 3.0
560 mg/L (start – 24 h): 2.8 – 2.7
Dissolved oxygen: 6.2 – 10.4 mg O_2/L
Conductivity: 281 µS/cm³ in the dilution water
Hardness: 56.3 mg $CaCO_3$/L

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity test was performed at nominal concentrations of 32, 56, 100, 180, 320 and 560 mg glyphosate acid/L prepared using filtered and dechlorinated tap water treated with ultra violet steriliser. The test was conducted under static test conditions. A negative control (dilution water only) was also prepared. A single replicate vessel was prepared for the control and at each treatment level, each containing ten fish (added to 40 L glass aquariums containing 30 L test medium).

2. Observations: Fish in all vessels were observed for sublethal effects and mortality after 24, 48, 72 and 96 hours. Temperature, pH-value and oxygen saturation of test solutions were measured on a daily basis. Hardness and conductivity of the test water was measured at test initiation. At test termination, the ten fish from the dilution water control were weighed and measured. Analytical measurements were performed by HPLC analysis at test initiation and after 48 and 96 hours.

3. Statistical calculations: The LC_{50} values and their 95% confidence intervals were calculated using non-linear interpolation. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: The mean measured concentrations of glyphosate acid ranged from 91 to 100%. As the measured concentrations of glyphosate were between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The 96 hour LC_{50} and NOEC values are presented below.

Endpoints	Glyphosate acid [mg/L]
LC_{50} (95% C.L.) (96 h)	130 (100 – 180)
NOEC (96 h)	32

B. OBSERVATIONS

Until 100 mg test item/L no mortality occurred, but all fish died at the test concentrations of 180 mg test item/L and higher. Sublethal effects like dark discolouration and loss of balance were observed at 56 and 100 mg test item/L respectively.

All measured water quality parameters were within the specifications recommended by the OECD 203 test guideline, except pH, where the levels of pH declined with increasing concentration of the test item. At 180 mg test item/L, the pH was 3.5 and lower.

The biological observations recorded during the test are presented in Table 8.2.1-2.

Table 8.2.1-2: Effects of glyphosate acid to rainbow trout

Nominal concentration of glyphosate acid [mg/L]	Number of dead fish / number of fish with intoxication symptoms [1] and observed symptoms			
	24 h	48 h	72 h	96 h
Control	0 / 0	0 / 0	0 / 0	0 / 0
32	0 / 0	0 / 0	0 / 0	0 / 0
56	0 / 0	0 / 0 DC	0 / 0 DC	0 / 0
100	0 / 0 DC	0 / 0 DC, LB	0 / 0	0 / 0
180	* / *	* / *	* / *	* / *
320	* / *	* / *	* / *	* / *
560	* / *	* / *	* / *	* / *

1 Dead fish are added to the sum of fish with symptoms
* / * All fish dead
DC Dark colouration; LB: Loss of balance

All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

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III. CONCLUSION

The 96 hour LC_{50} value for rainbow trout (*Oncorhynchus mykiss*) exposed to glyphosate acid was calculated to be 130 mg glyphosate acid/L (nominal) with 95% confidence interval of 100 to 180 mg/L. The NOEC after 96 h was 32 mg glyphosate acid/L.

Annex point	Author(s)	Year	Study title
IIA 8.2.1/02		1995	Glyphosate acid: Acute Toxicity to Bluegill Sunfish (*Lepomis macrochirus*) Report No: 5553/B Date: 1995-12-21 GLP: yes not published

Guideline: FIFRA Guideline 72-1

Deviations to OECD 203: None

Dates of experimental work: 1995-11-20 to 1995-11-24

Executive Summary

The effects of glyphosate acid to bluegill sunfish (*Lepomis macrochirus*) was evaluated in a 96-hour static toxicity test conducted at nominal test concentrations of 10, 18, 32, 56, 100 and 180 mg test item/L. A negative control (dilution water only) was prepared in parallel. Ten fish were exposed in the control and at each treatment level.

All fish were observed at daily intervals for the 96 hour study duration with mortality and sub-lethal signs of toxicity recorded. Dissolved oxygen, pH and temperature were also measured daily in each test vessel. Samples of control and test media were analysed for glyphosate acid at 0 hours (before fish addition) and after 48 and 96 hours. Glyphosate acid was not detected in the control group. Overall mean measured concentrations of glyphosate acid in test groups ranged from 94.4 to 97.0% of nominal concentrations.

There was no fish mortality in the control and at 10, 18 and 32 mg/L. At 56, 100 and 180 mg/L there was 90, 100 and 100% mortality respectively. There was a negative correlation between pH and test concentration.

Up to a nominal concentration of 32 mg test item/L, no mortality occurred during the 96 h exposure time. At the next higher concentration of 56 mg test item/L, 90% of fish died. At or above a concentration of 100 mg test item/L, while a strong correlation between pH value and test item concentrations was observed. At 56 mg test item/L, the pH was reduced to 3.8 and lower. All validity criteria according to the OECD guideline 203 were fulfilled.

The 96 hour LC_{50} value for bluegill sunfish (*Lepomis macrochirus)* exposed to glyphosate acid was 47 mg glyphosate acid/L (nominal) with 95% confidence interval of 35 to 66 mg/L. The NOEC after 96 hours was 32 mg glyphosate acid/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6% a.s.
2. Vehicle and/or positive control:	Filtered and dechlorinated tap water
3. Test organism:	
Species:	Bluegill sunfish (*Lepomis macrochirus*)
Age:	Juvenile
Size:	30 mm (mean)
Body weight:	0.54 g (mean)
Loading:	10 test individuals for 20 L test solution
Source:	[redacted] USA
Diet/Food:	no feeding for 48 hours prior to test and during the total test period
Acclimation period:	19 days at 22°C prior to the test initiation
4. Environmental conditions:	
Temperature:	22 ± 1°C
Photoperiod:	16 hours with 20 min transition period
pH:	Control (start – 96 h): 7.3–6.8 10 mg/L (start – 96 h): 5.9 – 6.4 18 mg/L (start – 96 h): 5.2 – 5.8 32 mg/L (start – 96 h): 4.6 – 4.8 56 mg/L(start – 96 h): 3.8 – 3.9 100 mg/L (start – 24 h): 3.4 180 mg/L (start – 24 h): 3.1
Dissolved oxygen:	6.2 – 9.0 mg/L
Conductivity:	100 µS/cm
Hardness:	16.0 mg $CaCO_3$/L.

B. STUDY DESIGN AND METHODS

1. Experimental treatments: The acute toxicity test was performed at nominal concentrations of 10, 18, 32, 56, 100 and 180 mg test item/L prepared using filtered and dechlorinated tap water treated with ultra violet steriliser. The test was conducted under static test conditions (no media renewal). A negative control group (dilution water only) was also prepared. A single vessel was prepared for the control and each test media group, each containing ten fish (27.5 L borosilicate glass vessels containing 20 L test medium).

2. Observations: All fish were observed for sublethal effects and mortality after 24, 48, 72 and 96 hours. Temperature, pH-value and oxygen saturation of test solutions were measured on a daily basis. Hardness

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and conductivity of the test water was measured at test initiation. Samples of test media were analysed for glyphosate acid content using HPLC analysis at test initiation and after 48 and 96 hours.

3. Statistical calculations: The 96 hour LC_{50} values and 95% confidence intervals were calculated using non-linear interpolation. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: The measured concentrations of glyphosate acid in fresh media at test initiation ranged between 96.9 and 110% of nominal. In aged test media at 96 hours, mean measured glyphosate acid concentrations ranged between 94.4 and 97.0% of nominal. At 100 and 180 mg/L, no chemical analysis was performed at 48 and 96 hours, as all fish died within the first 24 hours following addition.

As measured concentrations of glyphosate acid were between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The 96 h LC_{50} value and corresponding NOEC value based on nominal concentrations are given below.

Endpoints	Glyphosate acid [mg/L]
96 hour LC_{50} (95% C.L.)	47 (35-66)
96 hour NOEC	32

B. OBSERVATIONS

There were no mortalities in the control or the 10, 18 and 32 mg /L treatments. At 56 mg test item/L, there was 90% mortality. There was 100% mortality at 100 mg/L and higher test concentrations that occurred after 24 hours. There was a strong negative correlation between pH value and test item concentrations observed. At 56 mg test item/L, the pH was reduced to 3.8 and lower.

The biological observations recorded during the test are presented in Table 8.2.1-3.

Table 8.2.1-3: Effects of glyphosate acid to *Lepomis macrochirus*

Nominal concentration of glyphosate acid [mg/L]	Number of dead fish / number of fish with intoxication symptoms [1)] and observed symptoms			
	24 h	48 h	72 h	96 h
Control	0 / 0	0 / 0	0 / 0	0 / 0
10	0 / 0	0 / 0	0 / 0	0 / 0
18	0 / 0	0 / 0	0 / 0	0 / 0
32	0 / 0	0 / 0	0 / 0	0 / 0
56	4 / 4	8 / 8	9 / 9	9 / 9
100	* / *	* / *	* / *	* / *
180	* / *	* / *	* / *	* / *

[1] Dead fish are added to the sum of fish with symptoms
* / * All fish dead

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All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

III. CONCLUSION

The 96 hour LC_{50} value for bluegill sunfish (*Lepomis macrochirus*) exposed to glyphosate acid was 47 mg glyphosate acid/L (nominal) with a 95% confidence interval of 35 to 66 mg/L, with a 96 hour NOEC values of 32 mg glyphosate acid/L.

Annex point	Author(s)	Year	Study title
IIA 8.2.1/03	[redacted]	2000	Acute Toxicity of Glifosate Técnico Nurfarm to Zebrafish (*Brachydanio rerio*) [redacted] Report No: [redacted]-D61.47/99 Date: 2000-01-26 GLP: yes not published

Guideline: OECD Guideline 203 (1993)

Deviations: None

Dates of experimental work: 1999-10-18 to 1999-10-22

Executive Summary

The effects of glyphosate acid on zebra fish (*Danio rerio*) were evaluated in a 96-hour semi-static toxicity test (48 hour media renewal) conducted at nominal test concentrations of 10, 32, 56, 100, 180, 320 mg test item/L. A negative control group (dilution water only) was also prepared. Two vessels were prepared per control and test treatment, each containing ten fish.

Observations for sub-lethal effects and mortality were performed at 3, 24, 48, 72 and 96 hours after the start of the test. Dissolved oxygen, pH and temperature were measured and recorded daily in each test vessel. Glyphosate acid concentrations were measured in new and old control and test media on each day of the test. Glyphosate acid was not detected in the control group. Overall mean measured concentrations of glyphosate acid ranged between 95.9 and 108.8% of nominal concentrations.

During the 96 hour exposure period, at nominal concentrations up to 56 mg test item/L, there were no sub-lethal effects or mortality recorded. At the concentration of 100 mg test item/L, there was 15% mortality with hyperactivity observed in test fish at 48 hours onwards. At a concentration of 180 mg test item/L and above, there was 100% mortality observed after 24 hours. There was a strong negative correlation between pH value and test item concentration observed. At 100 mg test item/L, the pH was reduced to 4.8 and lower. All validity criteria according to the OECD guideline 203 were fulfilled.

The 96 hour LC_{50} for zebra fish (*Danio rerio*) exposed to glyphosate acid was calculated to be 122.91 mg glyphosate acid/L (nominal) with a 95% confidence interval of 111.97 to 134.92mg/L. The 96 hour NOEC was 56 mg glyphosate acid/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White powder
Lot/Batch #:	037-919-113
Purity:	95.0% a.s. (nominal), 95.49% a.s.(analysed)

2. Vehicle and/or positive control: Reconstituted water; Reference: Potassium dichromate ($K_2Cr_2O_7$)

3. Test organism:

Species:	Zebra fish (*Danio rerio*)
Age:	Not stated
Size:	Not stated
Body weight of the animals:	0.191 -0.239 g
Loading:	(0.38 to 1.44 g fish/L) based on 10 specimens exposed in 3 L test solution
Source:	[redacted]
Diet/Food:	no feeding during the total test period
Acclimation period:	72 h (to dilution water) prior to the test initiation (no feeding 24 h prior to test start and during the test)

4. Environmental conditions:

Temperature:	24.1 – 24.5°C
Photoperiod:	16 hours
pH:	Control (start – 96 h): 7.4 – 7.5 10 mg/L (start – 96 h): 7.3 – 7.1 32 mg/L (start – 96 h): 7.0 – 6.6 56 mg/L(start – 96 h): 6.5 – 5.3 100 mg/L (start – 96 h): 5.1– 4.8 180 mg/L (start – 24 h): 4.1 – 4.0 320 mg/L (start – 24 h): 3.5 – 3.6
Dissolved oxygen:	4.9 – 5.8 mg O_2/L (61.72 % - 73.06% of saturation value at 24.5°C)
Conductivity:	691 - 711 µS/cm
Hardness:	229.7 – 249.9 mg $CaCO_3$/L.

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finding test, a definite toxicity test was performed using nominal concentrations of 10, 32, 56, 100, 180, 320 mg test item/L in a semi-static test setup, with test media renewal after 48 hours. A negative control (reconstituted water only) was also prepared. There were two vessels per treatment, containing ten fish each (4000 mL glass vessels containing 3000 mL test medium).

2. Observations: All fish were observed for sublethal effects and mortality after 3, 24, 48, 72 and 96 hours. Temperature, pH-value and oxygen saturation of test solutions were measured on a daily basis. Weight measurements were conducted of each individual fish at test initiation. Samples of test media were analysed using HPLC analysis at test initiation and after 48 and 96 hours.

3. Statistical calculations: LC_{50} values, along with respective 95% confidence limits were calculated using the Trimmed Spearman-Karber Method. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Mean measured concentrations of glyphosate acid ranged between 95.9% and 108.8% of the nominal test concentrations. As values were between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal test item concentration.

The 96 h LC_{50} and corresponding NOEC values based on nominal concentrations are given below..

Endpoints	Glyphosate acid [mg/L]
96 hour LC_{50} (95% C.L.)	122.91 (111.97 – 134.92)
96 hour NOEC	56

B. OBSERVATIONS

At the 180 mg glyphosate acid/L concentrations and higher, 100% mortality was observed after 24 hours exposure. At 100 mg glyphosate acid/L, there was 20% mortality after 72 hours and 30% mortality after 96 hours, with hyperactivity observed in test fish at 48 hours onwards. At 56 mg test item/L and lower, no fish mortalities or sub-lethal effects were observed throughout the test period. .

The biological observations recorded during the test are presented in Table 8.2.1-4.

Table 8.2.1-4: Lethal effects of glyphosate acid to zebra fish

Nominal concentration of glyphosate acid [mg/L]	Number of dead fish and observed symptoms				
	3 h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
10	0	0	0	0	0
32	0	0	0	0	0
56	0	0	0	0	0
100	0	0	0 HA	2 HA	3 HA
180	0 LE	10	10	10	10
320	9 LE	10	10	10	10

[1] Dead fish are added to the sum of fish with symptoms
* All fish dead
LE loss of equilibrium
HA hyperactivity

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The 96 h LC_{50} (95% CL) for the reference product was calculated to be 79.54 (68.87 – 91.88) mg/L.

All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

III. CONCLUSION

The 96 hour LC_{50} for zebra fish (*Danio rerio*) exposed to glyphosate acid was 122.91 mg glyphosate acid/L (nominal) with a 95% confidence interval of 111.97 to 134.92mg/L. The 96 hour NOEC was 56 mg glyphosate acid/L.

Annex point	Author(s)	Year	Study title
IIA 8.2.1/04	[redacted]	2006	Glyphosate Technical: Acute Toxicity to Common Carp (*Cyprinus carpio*) [redacted] Report No: 2060/015 Date: 2006-01-03 GLP: Yes not published

Guideline: OECD Guideline 203 (1992); JMAFF Testing Guideline for Toxicology Studies, 12 NohSan No. 8147, Guideline 2-7-1(2000)

Deviations to OECD 203: None

Dates of experimental work: 2005-05-31 to 2005-06-04

Executive Summary

The effects of glyphosate acid to common carp (*Cyprinus carpio*) were evaluated in a 96-hour semi-static toxicity test (48 hour renewal of test media) conducted as limit test at a nominal test concentration of 100 mg test item/L. A negative control (dilution water only) was prepared in parallel. Duplicate control and test vessels were prepared, each containing seven fish.

All fish were observed for sub-lethal effects and mortality at 3, 6, 24, 48, 72 and 96 hours after the start of the test (fish addition). Dissolved oxygen, pH and temperature were measured and recorded daily in each test vessel. Glyphosate acid concentrations were measured at 0, 24 and 96 hours. Glyphosate acid was not detected in the control group. Mean measured concentrations ranged from 90 to 98% of nominal concentrations.

At the nominal concentration of 100 mg test item/L, there was no mortality or sub-lethal effects observed. All validity criteria according to the guideline OECD 203 were fulfilled.

Glyphosate acid resulted in no mortality or sub-lethal effects in common carp at 100 mg a.s./L. The 96 h LC_{50} value for common carp exposed to glyphosate acid was determined to be > 100 mg a.s./L, the highest concentration tested. The NOEC was 100 mg glyphosate acid/L.

I. MATERIALS AND METHODS

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A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White crystalline solid
Lot/Batch #:	H05H016A
Purity:	95.7%

2. Vehicle and/or positive control:

Dechlorinated tap water; Pentachlorophenol sodium salt (reference, tested in a different study)

3. Test organism:

Species:	Common carp (*Cyprinus carpio*)
Age:	Juvenile
Size:	4.2 ± 0.1 cm
Body weight:	2.05 ± 0.13 g
Loading:	0.72 g body weight/L test solution
Source:	UK
Diet/Food:	no feeding during the total test period
Acclimation period:	12 days at test conditions

4. Environmental conditions:

Temperature:	20.6 – 21.2°C
Photoperiod:	16 hours light / 8 hours dark, with 20 minutes dawn and dusk transition
pH:	7.4 – 8.3 (control), 6.3 – 8.0 (treatment)
Dissolved oxygen:	8.1 - 8.8 mg/L (91 - 99% saturation at 20.6 – 21.2°C)
Conductivity:	359 – 610 µS/cm
Hardness:	Approx. 100 mg $CaCO_3$/L.

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finding test, a final toxicity test was performed under semi-static test design as limit test using a single nominal concentration of 100 mg test item/L. The control and test media at 100 mg/L were renewed at 48 hours. A negative control group (dilution water only) was also prepared in parallel. There were duplicate glass vessels for the test concentration and control, each containing seven test fish in 20 L test medium.

2. Observations: All fish were observed for sub-lethal effects and mortality after 3, 6, 24, 48, 72 and 96 hours after test initiation (fish addition). Test solutions were renewed after 48 hours. Water temperature, pH-value and oxygen saturation of the test solutions were measured on a daily basis. Water hardness was measured in fresh media only. Samples of fresh media were taken at o hours and samples of old test media were taken at 24 and 96 hours to be analysed for glyphosate using a HPLC method of analysis.

3. Statistical calculations: Since the mortality was < 50%, no statistical calculation of LC_{50} values was possible. Therefore, NOEC and LC_{50} were determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Mean measured test item concentrations ranged from 90% to 98% of nominal test concentration. Therefore, endpoints were evaluated using nominal test item concentrations.

The 96 h LC_{50} and corresponding NOEC values based on nominal concentrations are given below.

Endpoints	Glyphosate acid [mg/L]
96 hour LC_{50}	> 100
96 hour NOEC	100

Reference test: The 96 h LC_{50} for the reference item pentachlorophenol was 0.26 mg/L, which is within the normal range of the reference material. The reference item was tested in a separate study.

B. OBSERVATIONS

At the 100 mg glyphosate acid/L concentration, there was no mortality during the 96 hours of exposure. In addition, no sub-lethal effects were observed.

All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

III. CONCLUSION

The 96 h LC_{50} for common carp (*Cyprinus carpio*) exposed to glyphosate acid in a limit test was determined to be > 100 mg a.s./L, with a 96 hour NOEC of 100 mg glyphosate a.s./L

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Annex point	Author(s)	Year	Study title
IIA 8.2.1/05	[redacted]	1998	96-Hour Acute Toxicity Study in Rainbow trout with (Aminomethyl)Phosphonic Acid (Static). [redacted] Report No: 232469 Date: 1998-06-29 GLP: yes Not published

Guideline: EEC directive 92/69, Part C.1
OECD guidelines No. 203 (1992).

Deviations to OECD 203: None

Dates of experimental work: 1998-05-14to 1998-05-29

Executive Summary

The toxicity of AMPA (aminomethyl)phosphonic acid) on rainbow trout (*Oncorhynchus mykiss*) was determined in a 96-hour static toxicity test conducted as a limit test at a nominal test concentration of 100

mg AMPA /L. A negative control (dilution water only) was prepared in parallel. Seven fish were added to the control and each AMPA treated vessel.

Observations for sub-lethal effects and mortality were performed at 2, 24, 48, 72 and 96 hours after the start of the test (fish addition). Dissolved oxygen, pH and temperature were measured and recorded daily in each test chamber. AMPA concentrations were measured at 0 (freshly prepared test media before fish addition) and 96 hours (test end). AMPA was not detected in the control group. Mean measured concentrations ranged between 97 to 105% of nominal concentrations. Toxicity was evaluated based on the nominal concentrations.

There were no sub-lethal effects of fish mortalities observed at the nominal 100 mg AMPA/L concentration during the 96 h exposure time. All validity criteria according to the guideline OECD 203 were fulfilled.

The 96 h LC_{50} for rainbow trout (*Oncorhynchus mykiss*) exposed to AMPA in a limit test was determined to be > 100 mg/L. The 96 hour NOEC was 100 mg AMPA/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: (Aminomethyl)phosphonic acid (AMPA)
Description: White powder
Lot/Batch #: A010047101
Purity: 99%

2. Vehicle and/or positive control: Tap water; Reference item: Pentachlorophenol

3. Test organism:

Species: Rainbow trout (*Oncorhynchus mykiss*)
Age: Juveniles
Size: 4.14 ± 0.34 cm
Body weight of the animals: 0.54 ± 0.20 g (mean weight of 10 representative individuals)
Loading: 0.38 g fish/L (7 fish per 10 L of test medium)
Source: [redacted] Netherlands.
Diet/Food: Last feeding at about 30 hours prior to test initiation and no feeding during the test period
Acclimation period: At least 12 days after delivery

4. Environmental conditions:

Temperature: 14.2 – 14.8°C
Photoperiod: 16 hours light
pH: 7.3 – 8.4
Dissolved oxygen: 9.3 – 9.7 mg O_2/L
Conductivity: Not stated
Hardness: 2.4 mmol/L

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The test was conducted as a static (without renewal) 96 hr limit test at a nominal test concentration of 100 mg AMPA/L, based on the results of a range finding test. The test media was prepared by direct addition of AMPA to tap water. A negative control (dilution water only) was prepared in parallel. Single vessels (18-L glass aquariums) containing 10 litres of control, or test media were prepared. Seven fish were added to each vessel at the start of the test.

2. Observations: All fish were observed for sub-lethal effects and mortalities after 2, 24, 48, 72 and 96 hours. Temperature, pH-value and oxygen saturation of the test solutions were measured on a daily basis. Hardness of the test water was measured at test initiation only.

Prior to the start of the test, ten representative fish from the fish stock used in the test were weighed (wet weight (g)) and measured (total length (cm)).

Samples of control or test media were taken at test start (0 hours) before fish addition and at 96 hours (test end). Concentrations of AMPA in each sample were determined using an HPLC method of analysis.

3. Statistical calculations: Since the mortality was < 50%, no statistical calculation of LC_{50} values was possible. Therefore, NOEC and LC_{50} were determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Measured concentrations of AMPA in media samples taken at the start of the test before fish introduction were 105% of nominal. At the end of the test, concentrations in the aged test media were 97% of nominal.

The mean measured concentration of AMPA ranged between 80 and 120% of nominal, therefore the ecotoxicological endpoints were evaluated based on the nominal AMPA concentrations.

The 96 hour LC_{50} and NOEC values for rainbow trout exposed to AMPA are given below.

Endpoints	Aminomethylphosphonic acid (AMPA) [mg/L]
96 hour LC_{50}	> 100
96 hour NOEC	100

Reference test: The determined 96h-LC_{50} for the reference item pentachlorophenol was 0.30 mg/L, which correspond well with the historical range of 0.10 - 0.46 mg/L. Thus, the sensitivity of trout from the present batch corresponded with the historical data.

B. OBSERVATIONS

There were no sub-lethal effects or mortality observed in fish exposed to AMPA during the 96 hours limit test at 100 mg AMPA/L.

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All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

III. CONCLUSION

The 96 h LC_{50} for rainbow trout exposed to AMPA was determined to be >100 mg/L. The 96 hour NOEC was considered to be 100 mg AMPA/L, the maximum concentration tested.

Annex point	Author(s)	Year	Study title
IIA 8.2.1/07	[redacted]	2003	MON 78623: A 96-hour Static Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*) [redacted] Report No: [redacted]-2002-149 Date: 2003-08-28 GLP: no not published

Guideline: OECD Guideline 203; OPPTS 850.1075; EU Guideline C1

Deviations to OECD 203: The temperature was lower than recommended (12.2 – 12.7 °C instead of the recommended 13 – 17 °C), since it has been found to be an acceptable temperature to maintain healthy rainbow trout.

Dates of experimental work: 2003-02-21 to 2003-02-25

Executive Summary

The toxicity of glyphosate potassium (K) salt on rainbow trout (*Oncorhynchus mykiss*) was determined in a 96-hour static (without media renewal) toxicity test conducted at nominal test concentrations of 156, 313, 625, 1250 and 2500 glyphosate K-salt /L. A negative control group (dilution water only) was also prepared. Duplicate vessels were prepared for the control and each test item level, with 10 fish added to each vessel.

Observations for sub-lethal effects and mortality were performed at 4, 24, 48, 72 and 96 hours after the start of the test (fish addition). The pH-value and oxygen saturation of the test solutions were measured at test initiation and at daily intervals. Temperature was measured at test initiation and termination. Samples of test media were taken at the start (before fish addition), and after 48 and 96 hours for the analysis of glyphosate K salt using an HPLC method of analysis. Overall mean measured glyphosate K salt concentrations were 159, 329, 646, 1302 and 2573 mg glyphosate K-salt /L. Glyphosate K-salt was not detected in the control group. Mean measured concentrations ranged from 99.8 to 109% of nominal concentrations. Toxicity evaluations were based on measured concentrations.

There was no mortality in the control, 159, 329 and 646 mg glyphosate K salt/L treatment groups. In the 1302 and 2573 mg glyphosate K salt/L treatment groups, there was 5 and 15%, respectively, with significant sub-lethal effects (including erratic swimming, and loss of equilibrium) observed in the 646, 1302 and 2573 mg glyphosate K salt/L treatment groups within 15 minutes of fish addition. Test media pH was negatively correlated with test concentration. All validity criteria according to the guideline OECD 203 were fulfilled.

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The 96 hour LC_{50} for rainbow trout (*Oncorhynchus mykiss*) exposed to glyphosate K-salt was determined to be > 2573 mg /L, equivalent to >1227.3 mg a.s./L. The 96 hour NOEC was determined to be 329 mg glyphosate K-salt /L, equivalent to 156.9 mg a.s./L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 78623 (Glyphosate K-salt)
Description:	yellow liquid
Lot/Batch #:	GLP-0108-11688-F
Purity:	47.7%
2. Vehicle and/or positive control:	Dechlorinated and filtered tap water
3. Test organism:	
Species:	Rainbow trout (*Oncorhynchus mykiss*)
Age:	Juvenile
Size (mean standard length):	43 mm (38 – 56 mm)
Weight (mean wet weight):	0.94 g (0.59 – 1.3 g)
Loading:	0.47 g fish/L
Source:	USA
Acclimation period:	5 weeks prior to the test initiation
4. Environmental conditions:	
Temperature:	12.2 – 12.7°C
Photoperiod:	16 h light, with a 30 min transition period
pH:	Control (start – 96 h): 8.2 – 8.0 156 mg/L (start – 96 h): 7.5 – 8.1 313 mg/L (start – 96 h): 7.1 – 8.0 625 mg/L (start – 96 h): 6.7 – 7.9 1250 mg/L (start – 96 h): 6.2 – 7.1 2500 mg/L (start – 96 h): 5.7 – 5.8
Dissolved oxygen:	≥ 7.3 mg/L (≥ 67% saturation)
Conductivity:	280 µS/cm
Hardness:	144 mg $CaCO_3$/L.
Alkalinity:	184 mg $CaCO_3$/L

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A definite toxicity test was performed using nominal concentrations of 156, 313, 625, 1250 and 2500 mg test item/L (mean measured: 159, 329, 646, 1302 and 2573 mg test item/L) in a static test setup, based on the results of a range finding test,. A negative control group (dilution water only) was prepared in parallel. Duplicate vessels (38 L glass vessels containing 20-L control water or test medium) were prepared for the control and treatment groups, each containing ten fish (20 fish per treatment).

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2. Observations: Observations for sub-lethal effects and mortality were performed at 4, 24, 48, 72 and 96 hours after test initiation (fish addition). The pH-value and oxygen saturation of the test solutions were measured at test initiation and on each observation date. Temperature was measured at test initiation and termination. Hardness, alkalinity and specific conductivity of the test water were measured at the start of the test only. Fish wet weights and total lengths were measured in the control. Samples of control or test media from all vessels was taken at 0 (before fish addition) 48 and 96 hours and analysed to determine the to measure glyphosate K salt concentration.

3. Statistical calculations: Since the mortality was < 50%, no statistical calculation of LC_{50} values was possible. The NOEC was determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Chemical analyses were performed on samples of the test solutions to quantify glyphosate concentrations in the test solution. Measured concentrations were between 99.8 and 109% of nominal confirming the stability of the test substance in the test system. The ecotoxicological endpoints are based on the mean measured concentrations of 159, 329, 646, 1302 and 2573 mg glyphosate K salt /L, (equivalent to 75.8, 156.9, 308.1, 621.1 and 1227.3 mg glyphosate acid/L).

The 96 hour LC_{50} and NOEC values for rainbow trout (*Oncorhynchus mykiss*) exposure to glyphosate K salt are given below.

Endpoints	Expressed as Glyphosate K-salt [mg/L]	Expressed as Glyphosate acid [mg a.s./L]
96 hr LC_{50}	> 2573	1227.3
96 hr NOEC	329	156.9

B. OBSERVATIONS

There was no mortality or sub-lethal effects in the negative control and at the mean measured concentrations of 159 and 329 mg glyphosate K salt/L. At 1302, 646 and 2573 mg glyphosate K salt/L, 0, 5 and 15% mortality were observed respectively.

At the three highest test concentrations, sub-lethal effects were noted within 15 minutes after test initiation (including surfacing, laying on the bottom of test chamber, erratic swimming, loss of equilibrium).

The severity of effect generally increased with increasing concentration, which correlated to the concentration-responsive decrease in pH. The pH at 0 h decreased from 8.2 for the controls to 5.7 at the highest test concentration. All surviving fish in 646 and 1302 mg test item/L appeared normal by 24 h and appeared normal for the remainder of the test. Effects were still evident in three of the 17 surviving fish in 2573 mg test item/L at test termination. The pH remained below 6 in the highest test concentration throughout the test.

The biological results achieved during the fish acute toxicity test are presented in Table 8.2.1-5.

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Table 8.2.1-5: Lethal effects of glyphosate K-salt to rainbow trout (*Oncorhynchus mykiss*)

Glyphosate K-salt [mg/L]	Glyphosate acid [mg a.s./L]	Number of dead fish / number of fish with intoxication symptoms and observed symptoms					
		0 h	**4 h**	**24 h**	**48 h**	**72 h**	**96 h**
Control		0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
159	**75.8**	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
329	**156.9**	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
646	**308.1**	0 / 0	0 / 20 A	0 / 11 A	0 / 0	0 / 0	0 / 0
1302	**621.1**	0 / 3 R / 17 E,N	1 / 17 A / 2R	1 / 0	1 / 0	1 / 0	1 / 0
2573	**1227.3**	0 / 8 R / 12 E,N	0 / 7 R / 13 A,E,N	0 / 6 R / 4 A / 2 E,N	3 / 0	3 / 3 R / 1 C	3 / 3 R

A = surfacing; R= laying at bottom of test chamber; E = erratic swimming, N = loss of equilibrium

All validity criteria according to OECD 203 were fulfilled, as mortality in control group did not exceed 10% (or one fish if less than ten are used), dissolved oxygen concentration was ≥ 60% of air saturation and constant exposure conditions have been maintained.

III. CONCLUSION

The 96 hour LC_{50} for rainbow trout (*Oncorhynchus mykiss*) exposed to the glyphosate K-salt was determined to be > 2573 mg /L, equivalent to >1227.3 mg a.s/L. The 96 hour NOEC was determined to be 329 mg glyphosate K-salt/L, equivalent to 156.9 mg a.s./L.

IIA 8.2.2 Chronic toxicity to fish

As summarized in the 2001 EU evaluation of glyphosate, glyphosate acid has low chronic toxicity to aquatic vertebrates. The chronic endpoint for the 255 day fish full life-cycle study with the fathead minnow was 25.7 mg a.s./L. Chronic NOEC values for fish ≥10 mg/L are indicative of low chronic toxicity. Supplementary chronic fish data included in this review confirms the low chronic toxicity of glyphosate to fish. A recently completed fish early life-stage study with AMPA demonstrates similarly low chronic toxicity to fish with a NOEC value of 9.63 mg a.s./L, which was the highest concentration tested. A summary of data reviewed in EU evaluation of glyphosate is included in Table 8.2.2-1.

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Table 8.2.2-1: Chronic toxicity of glyphosate acid and its metabolite AMPA to fish

Species	Test design	LC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/ GLP	2001 EU evaluation monograph reference
Glyphosate acid					
Danio rerio (fry)	168 h semi-static	24.71	18	IIA 8.2.3./01 -D61.16/99 2000/yes	NA
Oncorhynchus mykiss	21 d flow-through	>100	52	-89-36 1989/yes	94-00018
Oncorhynchus mykiss [1]	21 d flow-through	>150	150	271620 1990/yes	94-00015
Oncorhynchus mykiss [1]	85 d ELS flow-through	>9.63	9.63	IIA 8.2.4./01 1005.029.321 2010/yes	NA
Pimephales promelas [1]	255 d FLC flow-through	>25.7	**25.7**	75-129 1975/no	95-00020
Lepomis macrochirus	56 d bio-concentration flow-through	BCF = 1.1 ± 0.61 steady state after 120 ± 59 d		95-00725	#36440 (Part 1) 1989/yes 9303 (Part 2) 1989/yes
Glyphosate-IPA salt					
Oncorhynchus mykiss	21 d semi-static	2641	235.3	80-91-2328-04-93 1993/yes	95-00548
AMPA					
Pimephales promelas [1]	33 d ELS flow-through	-	12.0	IIA 8.2.4./02 -2010-328 2011/yes	NA

[1] Limit test, highest concentration tested
Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)
NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

IIA 8.2.3 Chronic toxicity (28 day exposure) to juvenile fish growth and behaviour

Annex point 8.2.3 refers to a 28-day growth and development exposure study. A 28-day growth and development study (OECD 215) was not performed and is not part of the EFSA aquatic guidance document. However, two older 21 day studies are summarised in Table 8.2.2 1 and were part of the 2001 EU glyphosate evaluation.

IIA 8.2.4 Fish early life stage toxicity test

In addition to a fish early stage study, a study is available for a short-term toxicity test on embryo and sac fry. This is not a traditional early life stage study because it is only 7 days in duration and it is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. The results of this study is superseded by the fish full life cycle study which has two full early life stage studies included in its experimental design; the first early life stage at the start of the test and the second early life stage after reproduction as the final phase of the study.

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Annex point	Author(s)	Year	Study title
IIA 8.2.3/01		2000	Chronic Toxicity of Glifosate Técnico Nufarm to Zebrafish larvae (*Brachydanio rerio*) Report No: D62.16/99 Date: 2000-01-13 GLP: yes Unpublished

Guideline: IBAMA 1990: Manual de testes para avaliacao da ecotoxicidade de agentes quimicos and OECD 212.

Deviations to OECD 212: Active ingredient concentrations were determined in the stock solutions only. Survival of fertilised eggs and differences of water temperature between test chambers or successive days is not reported.

Dates of experimental work: 1999-11-03 to 1999-11-10

Executive Summary

A fish short term toxicity test with glyphosate acid with larvae of *Danio rerio* (formerly named *Brachydanio rerio*) was performed under semi-static conditions. Three replicates with 30 fish per concentration were exposed for 168 hours to seven concentrations of glyphosate acid, ranging from 0.32 to 32 mg a.s./L.A control treatment containing reconstituted water and a toxic reference using potassium dichromate was maintained concurrently.

Observations for mortality and sub lethal responses were made every 24 hours. Dissolved oxygen, pH and temperature were measured and recorded daily. Glyphosate concentrations were measured by liquid chromatography in the stock solutions. Mean measured concentrations were at least 80% of nominal concentrations. Glyphosate acid was not detected in the control group.

A significant increase of mortality was observed at a concentration of 5.6, 10 and 32 mg a.s./L, behavioural responses such as lethargy was observed at 3.2, 5.6, 10 and 32 mg a.s./L. Several validity criteria according to the current OECD guideline 212 were not fulfilled as active ingredient concentrations were determined in the stock solutions only and survival of fertilised eggs and differences of water temperature between test chambers or successive days is not reported.

The No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC) for zebra fish larvae (*Danio rerio*) exposed to glyphosate acid were determined to be 3.2 mg a.s./L and 5.6 mg a.s./L, respectively, based on nominal concentrations. Chronic NOEC values >1 mg/L for aquatic invertebrates and vertebrates is categorized by Canton et al. (1991)[1] as practically nontoxic. The LC_{50} after 168 hours was determined to be 24.71 mg a.s./L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid

[1] Canton et al. 1991. Catch-up operation on old pesticides. National Institute of Public Health and Environmental Protection, Bilthoven. Report no. 678801001.

Lot/Batch #: 037-919-113

Purity: 954.9 g/kg acid equivalent

2. Vehicle and/or positive control: Tap water; Potassium dichromate ($K_2Cr_2O_7$)

3. Test organism:

Species: Zebra fish (*Danio rerio*) larvae

Age: Larvae, approx. 48 hours old

Size: Not stated

Loading: 1 L for 10 larvae

Source: Eggs: [redacted] Matrix fish: [redacted] [redacted] Brasil

Acclimation period: 48 hours prior to testing during embryo incubation and hatching

4. Environmental conditions:

Temperature: 23.8-24.3 °C

Photoperiod: 16 hours light / 8 hours dark

Dissolved oxygen: 60-100%

Conductivity of test medium: 168 µS/cm

Hardness of test medium: 44.1 mg/L $CaCO_3$

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The fish early life-stage toxicity test was performed under semi-static exposure conditions renewing the test solution every 48 hours. Following a range finding test, the freshly hatched fry of *Danio rerio* was exposed to test concentrations of 0.32, 0.56, 1.0, 3.2, 5.6, 10 and 32 mg glyphosate acid/L for 168 hours. A control consisting of reconstituted water and five toxic reference concentrations (32, 56, 100, 140 and 180 mg $K_2Cr_2O_7$/L were maintained concurrently.

2. Observations: Observations for mortality and sublethal responses were made every 24 hours. Dead individuals were removed at each observation. Temperature, dissolved oxygen, pH and conductivity were measured daily. The active ingredient analysis of stock solutions was performed by liquid chromatography.

3. Statistical calculations: LC_{50} and its confidence limits were determined using trimmed Spearman-Karber method. Fisher's Exact test was used for determination of significant differences in survival between control and exposure. The NOEC and LOEC were determined by visual interpretation of the mortality and observation data.

II. RESULTS AND DISCUSSION

A: FINDINGS

Analytical results: The active ingredient concentration in each stock solution was at least 80% of the nominal concentration throughout the test. Ecotoxicological relevant endpoints were therefore evaluated using nominal concentrations of the test item.

The 168 LC_{50}, NOEC and LOEC values are given below based on nominal concentrations.

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Endpoints	Glyphosate [mg a.s./L]
LC_{50} (168 h)	24.71 (95% C.I. 13.75 – 44.40 mg a.s./L)
LOEC (168 h)	5.6
NOEC (168 h)	3.2

B. OBSERVATIONS

A significant increase of mortality was observed at concentrations of 5.6, 10 and 32 mg a.s./L. Behavioural responses such as lethargy was observed at 3.2, 5.6, 10 and 32 mg a.s./L.

The results of the test are depicted in the following table.

Table 8.2.4-1: Lethal effects of glyphosate acid to zebra fish

Glyphosate acid[mg a.s./L]	C	0.32	0.56	1.0	3.2	5.6	10	32
Mortality (168 h) [%]	0	0	0	0	10	16.7	26.7	56.7

C Control

For the reference compound potassium dichromate ($K_2Cr_2O_7$) a 168 hour LC_{50} value of 124.66 mg/L (95% C.I. 112.08 – 138.67 mg/L) was determined.

With regard to the validity criteria of the pertaining OECD guideline 212, survival of fertilised eggs and differences of water temperature between test chambers or successive days is not reported. Mortality in control group did not exceed 10%, dissolved oxygen concentration was between 60 and 100% of air saturation. Analysis of test item treatments was performed only for the stock solutions.

III. CONCLUSION

In a short term toxicity test on fish larvae, the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC) for zebra fish (*Danio rerio*) exposed to glyphosate acid were determined to be 3.2 mg a.s./L and 5.6 mg a.s./L, respectively, based on nominal concentrations. Chronic NOEC values >1 mg/L for aquatic vertebrates is categorized by Canton et al. (1991)[2] as practically nontoxic. The LC_{50} after 168 hours was determined to be 24.71 mg a.s./L.

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[2] Canton et al. 1991. Catch-up operation on old pesticides. National Institute of Public Health and Environmental Protection, Bilthoven. Report no. 678801001.

Annex point	Author(s)	Year	Study title
IIA 8.2.4./01	[redacted]	2010	Glyphosate acid: Early life-stage toxicity test with rainbow trout (*Oncorhynchus mykiss*) under flow-through conditions [redacted] Report No: 1005.029.321 Date: December 23, 2010 GLP: yes Unpublished

Guideline: OECD Guideline 210 (1992)

Deviations to OECD 210: none

Dates of experimental work: May 14 to August 10, 2009

Executive Summary

The effects of glyphosate acid on the early life-stages of rainbow trout was determined under flow-through (continuous renewal) exposure conditions. Fertilized eggs of *Oncorhynchus mykiss* were exposed for 85 days to nominal glyphosate acid concentrations of 0.095, 0.305, 0.977, 3.125 and 10.0 mg/L. Initially, 50 fertilized eggs were exposed in duplicate exposure vessels at each of the five concentrations, with duplicate negative control groups (dilution water only) run in parallel.

Eggs were fertilized in the laboratory directly before addition to egg cups and remained undisturbed in the test system in the dark until hatching success was determined on days 22 to 26, based on the number of viable eggs. On day 26 (complete hatch), twenty fish fry per replicate i.e. 40 organisms per treatment level and control were transferred from egg cups to surrounding test media, where there development and survival was evaluated until test termination. Dissolved oxygen (DO) concentrations, pH and temperature were measured and recorded in each test vessel at experimental start and weekly thereafter until test termination (day 85). Glyphosate acid concentrations were measured on test days 0, 6, 13, 20, 27, 33, 41, 48, 55, 62, 70, 76 and 85. Glyphosate acid was not detected in the control group. Mean measured concentrations were substantially achieved and ranged between 85.7 and 96.3% of nominal concentrations. Ecotoxicological endpoint evaluation was based on overall mean measured glyphosate acid concentrations.

No statistical significant differences were detected for normal fry at hatch, hatching success, survival at test termination and growth (total length, wet and dry weight), when compared to the control group. All validity criteria according to OECD 210 were satisfied.

In a fish early life stage study performed with rainbow trout (*Oncorhynchus mykiss*) exposed to glyphosate acid, the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC) were determined to be 9.63 and > 9.63 mg glyphosate acid /L, respectively, based on mean measured concentrations.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid

Lot/Batch #: GLP-0807-19475-T

Purity: 96.03%

2. Vehicle and/or positive control: Reconstituted well water

3. Test organism:

Species: Rainbow trout (*Oncorhynchus mykiss*) eggs and milt

Age of eggs: Eggs and milt were less than 36 hours old at fertilization.

The time between fertilization and egg addition to test system was less than 3.5 hours

Number of animals/dose level: 40 organisms per replicate i.e. 40 organisms per treatment level and control

Supplier:

Mean loading rate (biomass per volume of test solution): 0.31 g/L per 24 hours

4. Environmental conditions:

Temperature: Continuously measured temperature: 9.4 to 13.1°C
Single-point measured temperature: 11.3 to 13.9ºC)

pH: 7.14 to 8.44

Dissolved oxygen: ≥60% ASV for study duration

Conductivity of test medium: 340 to 450 µS/cm

Hardness of test medium: 153 to 184 mg/L $CaCO_3$

Photoperiod: 16 hours with a 30 minute transition from Day 32 until test completion. Light intensity was 137 to 377 lux.

Eggs and larvae were shielded from all light during the incubation and hatching phases until one week after hatching

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The fish early life-stage toxicity test was performed under flow-through exposure conditions, using a constant-flow test item delivery system, supplying the appropriate test medium to duplicate exposure vessels at each of the five concentrations and the duplicate negative dilution water control vessels. The fertilized eggs were exposed to test concentrations of 0.095, 0.305, 0.977, 3.125 and 10.0 mg glyphosate acid/L for 85 days.

Twelve impartially located exposure vessels were maintained in a temperature-controlled water bath designed to maintain the test solution temperatures at 12 ± 2°C. During the egg exposure phase and until one week after hatching the test area was maintained in continuous darkness. From test day 32 until test completion, the vessels were illuminated to a light intensity of 137 to 377 lux using fluorescent tubes. A photoperiod of 16 hours was employed with a 30 minute (dawn/dusk) transition period.

Preparation of test solution: A 1 g glyphosate acid/L stock solution was prepared directly prior to test initiation and as required during the exposure period, by dissolving approximately 11.737 g of glyphosate acid in 10 L of dilution water. The stock solution was further diluted (dilution water) by the test item delivery system to achieve the required concentrations in each of the exposure vessels. For the control group, dilution water only without test item was used.

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Test units: The test vessels measured 39.0 cm x 19.2 cm, with an approximate water depth of 14.6 cm maintained at a constant volume of 10 L. Two replicates (A and B) were maintained for all treatments and the control.

Test initiation: Prior to fertilization, freshly collected rainbow trout milt and eggs were acclimatized in their respective delivery containers to the approximate test temperature of 12 ± 2°C, using a water bath and then mixed carefully together. The 'apparently' fertilized eggs were impartially distributed to egg incubation cups in groups of five, until each cup contained 50 eggs. The incubation cups were suspended in the respective exposure vessel with two cups per replicate vessel, resulting in 100 eggs per replicate. The test was initiated once all vessels contained eggs within 3.5 hours of receipt of the gametes and within two hours of fertilization.

Hatching success was determined on days 22 to 26 based on the number of viable eggs. Any eggs exhibiting embryonic development, whether dead or alive, at the time of assessment, were considered fertile for purposes of determining percent viability. All non-viable eggs were counted and discarded at day 26. The percent viability was calculated based on the actual number of fertilized embryos on day 26. Hatching success was calculated based on the actual number of viable embryos.

Egg exposure: Dead and alive eggs were counted daily. All eggs observed to be clear were considered to be alive, all eggs observed to be opaque and milky were considered to be dead. All eggs observed to be dead were removed and preserved in Stockard's solution for clearing and determination of embryonic development. Fry which hatched prior to the determination of viability were collected in an auxiliary egg cup.

Post hatch exposure: At completion of hatch on day 26, twenty organisms per replicate i.e. 40 organisms per treatment level and control were transferred directly from the first egg cup (i.e., A1 and B1) to the surrounding test media in the test vessels and the egg cups were removed.

For replicate A of the control and the 0.095 mg glyphosate acid/L treatment, 20 fry in the auxiliary egg cup containing the early hatched fry were randomly selected. For replicate A of the 10 mg a.s./L treatment, only eight viable eggs hatched of the 20 randomly selected eggs and therefore only eight hatched fry were released into the test vessel.

All remaining alive and dead eggs were preserved in Stockard's solution. The remaining fry were recorded and then discarded. After evaluation of the developmental status of the cleared eggs, the viability of all eggs was calculated.

During the post-hatch exposure period, developing fry in all vessels were observed daily; recording behaviour and appearance. Dead fry were removed during these observations. Survival was estimated daily throughout the post-hatch period. At 60-days post-hatch exposure (experimental completion), the percentage fish survival was calculated.

Fry feeding: At the beginning of fry swim-up, the fry were fed live brine shrimp nauplii (*Artemia salina*), harvested from hydrated cysts (24 to 36 hours post-hydration) three times per day. Fish were not fed during the 24 hours prior to study termination.

Length and weight: At day 60 post-hatch all of the surviving fish in each replicate vessel were euthanized with MS-222 (tricain methane-sulfonate), measured and weighed individually to determine fish total lengths and wet weights, respectively for each treatment.

2. Observations: The dissolved oxygen (DO) concentrations, pH and temperature were measured and recorded in each test vessel at experimental start and weekly thereafter until test termination (day 85). On test day 75, the DO levels decreased to between 6.31 to 7.50 mg O_2/L, so aeration was provided to each test vessel until test completion.

Temperature was continuously monitored in one replicate (replicate A of the control) throughout the study. Total hardness, alkalinity and specific conductivity were monitored at experimental start and on test days 5, 11, 19, 25, 32, 39, 46, 53, 61, 67, 74 and 81 in one replicate of the highest treatment level and the control during the exposure.

Analytical measurements: Prior to the start of the exposure phase, i.e., day -2, samples from one replicate of the treatment level solutions and control solutions were collected and analysed for the active ingredient. Results of the pre-test analyses were used to assess correct dosage of the system before test initiation.

During the in-life phase, water samples of approximately 10 mL were removed from both replicates of each treatment level and control on test days 0, 6, 13, 20, 27, 33, 41, 48, 55, 62, 70, 76 and 85 and the content of glyphosate acid was determined. Samples of the stock solutions were also analysed at each sampling interval.

Determination of NOEC and LOEC: Based on the results of statistical analysis performed for normal fry at hatch, hatching success, survival at test termination and growth (total length, wet and dry weight), the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC) were determined.

3. Statistical calculations: The data for percent normal fry at hatch, hatching success, survival at test termination and growth (total length, wet and dry weight) were first checked for normality using Shapiro-Wilks' Test (Weber *et al.*, 1989) and for homogeneity of variance using Bartlett's Test (Bartlett, 1937).

The data set for hatching success and survival at test termination were arc-sine (square root) transformed prior to determination of the NOEC and the LOEC by using one-way ANOVA and the parametric post-hoc Dunnett's Test (Dunnett, 1955, 1964). The data sets for growth passed the tests for homogeneity and normality, and Dunnett's Test was used to determine the NOEC and the LOEC.

II. RESULTS AND DISCUSSION

A: FINDINGS

The mean measured concentrations (calculated as geometric means) of 0.305, 0.977, 3.125 and 10.0 mg a.s./L ranged between 85.7 and 96.3% of the nominal test concentrations, with the exception of the lowest test concentration (0.095 mg a.s./L), where a mean recovery of 66.9% of the nominal concentration was calculated. Based on these results, the mean measured concentrations (calculated as geometric means) of 0.064, 0.261, 0.846, 2.804 and 9.63 mg a.s./L were used for the evaluation of the biological data.

The water quality parameters measured were not affected by test item concentrations. The results of the water quality measurements carried out during this study established that conditions maintained throughout the 85-day exposure were satisfactory for the promotion of normal rainbow trout embryo hatchability, fry survival and growth.

B. OBSERVATIONS

The effects of glyphosate acid on embryo viability, hatching success, number of normal fry at hatch, survival at test termination and growth (total length, wet and dry weight) are provided in Table 8.2.4-2 below.

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Table 8.2.4-2: Egg viability, hatching success and normal fry at completion of hatch (test day 26) and survival, total length, wet weight and dry weight of rainbow trout (*Oncorhynchus mykiss*) at test termination of the 85-day exposure to glyphosate acid.

Mean measured concentration (mg a.s./L)	Egg viability [%][A]	Hatching success [%][A]	Normal fry at hatch [%]	60 days post-hatch			
				Survival [%]	Total length [mm]	Wet weight [mg]	Dry weight [mg]
Control	35±3.3	92±6.9	97±0.56	85±7.1	46.38±0.41	942.6±34.9	195.1±14.3
0.064	43±4.9	84±20.2	96±5.2	95[B]±7.1	45.33±0.83	899.6±10.7	188.7±5.9
0.261	40±4.0	99±1.7	100±0.0	95±0.0	46.75±0.65	932.2±60.5	190.7±7.5
0.846	38±9.9	95±1.5	100±0.0	93±10.6	46.37±1.7	908.6±84.3	189.1±23.0
2.804	41±2.1	91±5.5	99±2.0	95±7.1	46.19±0.33	889.7±23.7	188.4±10.7
9.63	27±9.2	80±28.3	98±2.1	100±0.0	46.38±1.7	947.3±135	203.0±36.5

[A] Based on total number of viable eggs

[B] On test day 59, one fish of replicate A was inadvertently injured during the cleaning process of the test vessel. One day later this fish had died. Since this mortality was not test item related, the fish was therefore excluded from further statistical evaluation.

The NOEC and LOEC values for survival and growth of rainbow trout (*Oncorhynchus mykiss*) after 85-day exposure to glyphosate acid are based on mean measured concentrations.

Endpoint	Glyphosate acid [mg a.s./L]	
	NOEC	LOEC
Percent normal fry at hatch	9.63	>9.63
Hatching success	9.63	>9.63
Survival at test termination	9.63	>9.63
Total length	9.63	>9.63
Wet weight	9.63	>9.63
Dry weight	9.63	>9.63

All validity criteria according to OECD 210 were fulfilled, as dissolved oxygen concentration was between 60% and 100% of air saturation, water temperature was within the range specified for the test species and constant exposure conditions have been maintained (i.e. within ±20% of nominal concentration were recovered, except for the lowest concentration which does not affect the results of the study), and overall survival of fertilised eggs in the controls was greater than or equal to the limits defined in Annexes 3 and 6 of OECD 210.

III. CONCLUSION

In a 85-day (60 days post-hatch) chronic study with rainbow trout (*Oncorhynchus mykiss*) exposed to glyphosate acid, the NOEC and LOEC values for percent normal fry at hatch, hatching success, fry survival, length and weight were 9.63 and > 9.63 mg a.s./L, respectively, based on mean measured concentrations. Chronic NOEC values >1 mg/L for aquatic vertebrates is categorized by Canton et al. (1991)[3] as practically nontoxic.

[3] Canton et al. 1991. Catch-up operation on old pesticides. National Institute of Public Health and Environmental Protection, Bilthoven. Report no. 678801001.

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Annex point	Author(s)	Year	Study title
IIA 8.2.4./02		2011	AMPA (Aminomethylphosphonic acid): An early life-stage toxicity test with the fathead minnow (*Pimephales promelas*) Report No: 2010-328 Date: 2011-06-16 GLP: yes Unpublished

Guideline: OECD Guideline 210 (1992)
OPPTS 850.1400
ASTM E 1241-05

Deviations to OECD 210: None

Dates of experimental work: 2011-01-13 to 2011-03-02

Executive Summary

The effects of AMPA (aminomethyl-phosphonic acid) on the time of hatch, hatching success, survival and growth of fathead minnow (*Pimephales promelas*), was evaluated in a fish early life-stage toxicity test performed under flow-through exposure conditions, using a continuous flow test item delivery system. The appropriate test medium was supplied to four replicates at each of five concentrations and a negative control (dilution water only) group. The fertilized eggs were exposed to nominal test concentrations of 0.75, 1.5, 3.0, 6.0 and 12 mg AMPA/L for a 5 day hatching period followed by a 28 day post hatch growth period.

AMPA concentrations in test media were measured on day 0, 7, 14, 21, 28 and 33. Mean measured concentrations ranged from 82.5 to 117% of nominal concentrations. AMPA was not detected in the control group.

No significant differences in the time to hatch, hatching success, survival at test termination and growth (total length, wet and dry weight) were observed, when compared to the control. All validity criteria according to the current guideline OECD 210 were fulfilled.

In an fish early life stage test (OECD 210), performed using fathead minnows (*Pimephales promelas*) the No-Observed-Effect Concentration (NOEC) and the Lowest-Observed-Effect Concentration (LOEC) for fathead minnow (*Pimephales promelas*) exposed to AMPA were determined to be 12.0 and > 12.0 mg AMPA/L, respectively, based on mean measured concentrations.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	AMPA (Aminomethylphosphonic acid)
Lot/Batch #:	GLP-0908-19984-A
Purity:	98.7%

2. Vehicle and/or positive control: Moderately hard well water

3. Test organism:

Species: Fathead minnow (*Pimephales promelas*) embryos <24 hours

old

Age of eggs: <24 hours old

Number of animals/dose level: 20 organisms per replicate i.e. 80 organisms per treatment level and control

Supplier: USA

Mean loading rate (biomass per volume of test solution): 0.05 g fish/L per 24 hours; instantaneous loading at the end of test: 0.32 g fish/L

Diet/Food: live brine shrimp nauplii (*Artemia* sp.), USA

4. Environmental conditions:

Temperature: 25±1 °C

pH: 7.8 to 8.2

Dissolved oxygen: ≥89% of saturation (7.3 mg/L)

Conductivity of test medium: 361 - 395 µS/cm

Hardness of test medium: 132 - 140 mg/L $CaCO_3$

Photoperiod: 16 hours with a 30 minute transition period
Light intensity ≈ 296 lux,.

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The fish early life-stage toxicity test was performed under flow-through exposure conditions, using a constant-flow test item delivery system, supplying the appropriate test medium to the exposure vessels at each of the five concentrations and a negative control (dilution water only) group. The embryos of fathead minnow (*Pimephales promelas*) were exposed to test concentrations of 0.73, 1.5, 2.9, 6.0 and 12.0 mg AMPA/L for 33 days. The test was conducted in a temperature controlled environmental chamber. The test vessels were 9 L glass aquaria with a constant volume of 7 L of test solution. Embryos were held in incubation cups constructed from glass cylinders 50 mm in diameter with 425 µm nylon screen mesh. Four replicates vessels were maintained for all treatments and the control.

At test initiation, embryos <24 hours old were impartially distributed to incubation cups. After a hatching period of 5 days, larvae were released into test chambers. Newly hatched larvae were fed live brine shrimp nauplii (*Artemia sp.*) harvested from hydrated cysts 2 - 3 times per day.

2. Observations: During the first day of exposure, embryos were observed twice for mortality and fungal infection. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos was performed once per day. Once hatching had reached >90% in the control groups on day 5 of the test, the larvae were released into their respective test vessels and the post-hatch period began. During the 28-day post-hatch exposure period, the number of fry mortalities and numbers of individuals exhibiting clinical signs of toxicity or abnormal behaviour was recorded. From these observations, the time to hatch, hatching success, and post-hatch growth and survival were evaluated. On day 28 of the post-hatch exposure period – test termination, the total length for all surviving fish was measured to the nearest 1 mm using a metric ruler and wet and dry weights of all fish was measured to the nearest 0.1 mg using an analytical balance. Fish were euthanized (MS-222) and dried to constant weight in an oven at approximately 60°C for approximately 47 hours to establish fish dry weight data.

Dissolved oxygen, temperature and pH were measured in alternating replicates of each treatment and control group at the beginning of the test, weekly during the test, and at the end of the test. Hardness, alkalinity and specific conductance were measured in alternating replicates of the negative control

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(dilution water) and the highest concentration treatment group at the beginning of the test, weekly during the test and at the end of the test.

Analytical measurements were performed by HPLC analysis using UV detection. Water samples were collected from one test chamber of each treatment and control group four days prior to test initiation to confirm the operation of the diluter. Water samples were collected from alternating replicate test chambers of each treatment and control group on day 0, 7, 14, 21, 28 and 33 (test termination) to determine concentrations of the test substance in the test chambers. All samples were collected at mid-depth in the test chambers, placed in glass vials and analysed immediately.

3. Statistical calculations: Data were statistically tested using Chi-square and Fisher's Exact test (discrete-variable data; $\alpha = 0.05$) and Dunnett's t-test (one-tailed, normal distributed data; $\alpha = 0.05$). The NOEC and LOEC were determined by visual interpretation of the observation data.

II. RESULTS AND DISCUSSION

A: FINDINGS

Analytical data: Analytical measurements were performed on samples of representative test concentrations. Recoveries ranged from 82.5 % to 117% relative to nominal concentrations for all test concentrations.

The water quality parameters measured were not affected by test item concentrations. The results of the water quality measurements carried out during this study established that conditions maintained throughout the 33-day exposure were satisfactory for the promotion of normal fathead minnow embryo hatchability, fry survival and growth.

B. OBSERVATIONS

The effects of AMPA on embryo viability, hatching success and growth (total length, wet and dry weight) are provided in Table 8.2.4-3 below.

Table 8.2.4-3: Hatching success, larval survival and total length, wet weight and dry weight of fathead minnow (*Pimephales promelas*) at test termination of the 33-day exposure to AMPA.

Mean measured concentration [mg AMPA/L]	Hatching success [%]	Survival to day 28 post hatch [%]	Growth 28 days post-hatch		
			Mean total length [mm]	Mean wet weight [mg]	Mean dry weight [mg]
Control	99	91	25.2 ±0.57	112.0 ±11.5	24.1 ±1.4
0.73	100	91	25.2 ±0.27	120.7 ±7.4	24.6 ±1.0
1.5	100	93	25.5 ±0.39	119.3 ±14.2	24.9 ±2.1
2.9	100	90	25.7 ±0.62	117.4 ±3.8	23.5 ±0.42
6.0	100	91	25.4 ±0.22	117.4 ±4.2	23.6 ±0.70
12	99	92	26.2 ±0.62	135.2 ±11.0	26.5 ±2.9

The majority of the fish in the control group and in the AMPA treatment groups appeared normal throughout the test. Through Day 7 post-hatch, in the control group and in the AMPA treatment groups, a low frequency of larvae were noted as either weak, lying on the bottom of the test chambers, curled, or having a curled or curved spine/crooked spine. The frequency of curved/curled or curled spine/crooked spine

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observed in the treatment groups were comparable to historical frequencies observed in control treatments in early life-stage studies with fathead minnows performed at the test facility and consequently concluded to be not treatment related. Additionally, the frequencies of the occurrence of smaller fish visually observed in the control and treatment groups were comparable and consistent with the individual dry weight measurements.

The 33-day NOEC values are given below based on mean measured concentrations.

Endpoints	AMPA [mg/L]
LOEC (33 days) for hatching success, survival or growth	>12
NOEC (33 days) for hatching success, survival or growth	12

All validity criteria according to OECD 210 were fulfilled, as dissolved oxygen concentration was between 60% and 100% of air saturation, water temperature was within the range specified for the test species and constant exposure conditions have been maintained (i.e. within ±20% of nominal concentration were recovered), and overall survival of fertilised eggs/embryos in the controls was greater than or equal to the limits defined in Annexes 3 and 6 of OECD 210.

III. CONCLUSION

In a fish early life stage test (OECD 210) performed using fathead minnow (*Pimephales promelas*) exposed to AMPA, the NOEC and LOEC values for hatching success, fry survival, length and weight were 12 and >12 mg AMPA/L, respectively, based on mean measured concentrations.

IIA 8.2.5 Fish life cycle test

A fish full life cycle test was performed using *Pimephales promelas* (fathead minnow) and is included in the 2001 EU Evaluation of Glyphosate. Glyphosate water concentrations were indirectly evaluated by measuring ortho-phosphate and total phosphorous levels. No effect on survival, growth or reproduction of adult fathead minnow or progeny were observed when exposed to concentrations of up to 25.7 mg a.e./L for up to 8 months (255 days) (see Table 8.2.2-1).

IIA 8.2.6 Bioconcentration potential in fish

Bioconcentration studies examining uptake and elimination in bluegill, crayfish and molluscs have been performed for glyphosate and were reviewed in the 2001 EU evaluation. A fish-bioconcentration study is not required, due to the low Log_{POW}, which is below the trigger value of 3 (Log_{POW} = -3.2). However, a fish bioconcentration study has been conducted which achieved a bioconcentration factor of 1.1± 0.61, which is far below the Annex VI BCF trigger value of 1000. Therefore, a study is not necessary to determine bioaccumulation in aquatic non-target organisms.

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Bioconcentration factor (BCF)	BCF = 1.1 ± 0.61; steady state after 120 ± 59 d log P_{ow} of glyphosate acid and its metabolites was < 3, accumulation potential in aquatic non-target organisms is hence considered to be low
Annex VI Trigger for the bioconcentration factor	100
Clearance time CT_{50} CT_{90}	Not relevant
Level of residues (%) in organisms after the 14-day depuration phase	Not relevant

IIA 8.2.6.1 Bioconcentration potential of the active substance in fish

Please refer to IIA 8.2.6.1

IIA 8.2.6.2 Bioconcentration potential of metabolites, degradation and reaction products

Glyphosate acid has a log P_{OW} value of <-3.2 and its metabolite AMPA has a log P_{OW} of -5.18. ,Therefore, based on the low log P_{OW} values the potential for bioconcentration is negligible.

IIA 8.2.7 Aquatic bioavailability/biomagnifications/depuration

Further studies were not required according to Commission Document 7651l/VI/99-final dated 21 January 2002 and Directive 2001/99/EC dated 20 November 2001.

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IIA 8.3 Toxicity to aquatic species other than fish and aquatic species field testing

IIA 8.3.1 Acute toxicity to aquatic invertebrates

A summary of all available relevant and compliant data (including data already reviewed during the 2001 EU evaluation of glyphosate) for glyphosate, glyphosate salts and AMPA are included in Table 8.3.1-1. Additionally, a new study was performed with HMPA an aquatic metabolite of glyphosate.

Table 8.3.1-1: Acute toxicity of glyphosate acid, glyphosate IPA salt, K-salt and metabolites AMPA and HMPA to aquatic invertebrates

Species	Test design	EC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid					
Daphnia magna	48 h static	>100	100	141863 1995/yes	96-00067
Daphnia magna	48 h	**40**	18	-	95-00537 1995
Daphnia magna	48 h static	647.4	464.8	IIA 8.3.1.1/01 -78-201 1978/no	NA
Daphnia magna	48 h static	84.0	60.3	IIA 8.3.1.1/02 272968 1991/yes	NA
Daphnia magna	48 h static	420.6	179.6	IIA 8.3.1.1/03 -D51.017/00 2000/yes	NA
Daphnia magna	48 h static	130 >1000 (pH adjusted)	100 1000 (pH adjusted)	IIA 8.3.1.1./04 5551/B 1996/yes	NA
Glyphosate-IPA salt					
Daphnia magna	48 h	**930**	320	-	94-01160 1981
Daphnia magna	48 h	>1000		-	94-00549 1993/?
Daphnia magna [1]	48 h static	>61.6	61.6	IIA 8.3.1.1./05 83-91-0737-00-93 1994/yes	NA
Daphnia magna	48 h static	>470.9	470.9	IIA 8.3.1.1./06 -D51.017/00 2000/yes	NA
Glyphosate K-salt					
Daphnia magna	48 h static	>2582	312	IIA 8.3.1.1/07 -2002-150 2003/yes	NA
AMPA					

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Species	Test design	EC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
Daphnia magna	48 h static	**690**	320	-90-401 1991/yes	94-01163
Daphnia magna	48 h static	>180	180	5061/B 1994/yes	NA
Daphnia magna	48 h static	>100		IIA 8.3.1.1./08 232471 1995/yes	NA
Daphnia magna	48 h	>180	180		94-00500 1993
HMPA					
Daphnia magna	48 h static	>100	100	IIA 8.3.1.1./09 -2010-329 2011/yes	NA

[1] single dose limit test
Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)
NA: Not applicable; study was not reviewed as part of the 2001 EU evaluation of glyphosate

IIA 8.3.1.1 Acute toxicity (24 and 48 hour) for *Daphnia* preferably *Daphnia magna*

Several acute toxicity studies exist for *Daphnia magna*, which were not included in the previous EU-Evaluation of Glyphosate (2001). These studies are summarised below and provide consistent information on the low acute toxicity of glyphosate to aquatic invertebrates.

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1/01		1978	Acute Toxicity of Technical Glyphosate (AB-78-201) to *Daphnia magna* Report No: 78-201 Date: 1978-08-31 GLP: no not published

Guideline: Committee on Methods for Toxicity Tests with Aquatic Organisms

Deviations to OECD 202: No analytical dose verification, only 3 control replicates used

Dates of experimental work: 1978-08-29 to 1978-08-31

Executive Summary

The effects of glyphosate acid on *Daphnia magna* were evaluated in a 48-hour static toxicity test. A definitive toxicity test was performed using at nominal concentrations of 560, 650, 750, 870 and 1000 mg glyphosate acid/L, (purity corrected concentrations of 464.8, 539.5, 622.5, 722.1, and 830.0 mg

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glyphosate acid/L). In addition, a negative control group (dilution medium only) was also prepared.. There were three vessels per treatment and control, each containing ten daphnids.

The total number of immobile *Daphnia magna* was recorded at 24 h and 48 h after test initiation.

At 48 hours, there was 0, 3.3, 33.3, 100 and 100% immobility of *Daphnia* at the respective glyphosate acid concentrations The validity criteria according to the guideline OECD 202 were fulfilled; however, no analytical verification was conducted and only three control replicates were used.

The 48 h EC_{50} for *Daphnia magna* exposed to glyphosate was calculated to be 780 mg glyphosate acid/L (equivalent to purity corrected 647.4 mg glyphosate acid/L). The 48- hour no-effect level (NOEC) was determined to be 647 mg/L, (equivalent to purity corrected 464.8 mg glyphosate acid/L).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: XHI-162
Purity: 83.0%

2. Vehicle and/or positive control: Well water

3. Test organism:

Species: *Daphnia magna*
Age: Neonates (< 18 h old)
Loading: 10 specimens for 250 mL test solution
Source: [redacted]
Diet/Food: None
Acclimation period: None

4. Environmental conditions:

Temperature: 19 ± 1°C
Photoperiod: 16 hours light / 8 hours dark
pH: 8.0 (at test termination, not specified for which group)
Dissolved oxygen: 7.5 mg/L
Conductivity: Not stated
Hardness: > 250 mg $CaCO_3$/L.

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finding test, a definitive toxicity test was performed using nominal concentrations of 560, 650, 750, 870, 1000 mg test item/L, (purity corrected concentrations of 464.8, 539.5, 622.5, 722.1 and 830.0 mg glyphosate acid/L) in a static test setup. The test solutions were prepared using test facility well water (Dissolved oxygen = 8.6 mg/L, pH = 7.8, hardness > 250 mg $CaCO_3$/L.). In addition, a control group was exposed to dilution water (negative control). There were three replicates per treatment, each containing ten daphnids. Test chambers were 500 mL glass beakers containing 250 mL of test medium.

2. Observations: Total number of immobile *Daphnia magna* was recorded at 24 h and 48 h after the test initiation. Temperature, pH, and oxygen saturation of the test solutions were measured at the test termination. Hardness of the test water was measured at test initiation.

3. Statistical calculations: EC_{50} values were calculated along with the 95% confidence limits using Probit analysis.

II. RESULTS AND DISCUSSION

A. FINDINGS

The EC_{50} and NOEC values are given below based on purity corrected concentrations.

Endpoints (48 h)	Purity corrected [Glyphosate acid mg/L]
EC_{50} (95% C.I.)	647.4 (577.7 - 725.4)
NOEC	464.8

B. OBSERVATIONS

At and above purity corrected concentrations of 830 mg test item/L, 100% immobilisation was observed while no immobilisation was observed at the corrected concentration of 465 mg test item/L, 48 hours after the test initiation. At concentrations of 540 and 623 mg test item/L, immobilisation of 3.3% and 33.3% of specimens was observed.

Table 8.3.1.1-1: Effects of glyphosate acid to *Daphnia magna*

Purity corrected [Glyphosate acid mg/L]	-	464.8	539.5	622.5	722.1	830.0
Immobility (24 h) [%]	0	0	0	6.7	73.3	100
Immobility (48 h) [%]	0	0	3.3	33.3	100	100

All validity criteria according to OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels. However, no analytical verification was conducted and only three control replicates were used.

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III. CONCLUSION

The 48 h EC_{50} for *Daphnia magna* exposed to glyphosate was calculated to be 780 mg glyphosate acid/L (equivalent to purity corrected 647.4 mg glyphosate acid/L). The 48- hour no-effect level (NOEC) was determined to be 647 mg/L, (equivalent to purity corrected 464.8 mg glyphosate acid/L).

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1/02	████	1990	48-Hour Acute Toxicity of GLYPHOSATE TECHNICAL to *Daphnia magna* (OECD-Immobilization Test) ████ Report No: 272968 Date: 1990-11-09 GLP: yes Not published

Guideline: OECD Guideline 202 (1984)
EEC Directive 92/69, Part C.2

Deviations to OECD 202: The pH was not in a range of 6-9, but from 2.3 – 7.6.

Dates of experimental work: 1990-07-03 to 1990-07-05

Executive Summary

The toxicity of glyphosate acid on *Daphnia magna* were evaluated in a 48-hour static toxicity test. The test was performed using five nominal concentrations, 62.5, 125, 250, 500 and 1000 mg glyphosate acid/L. a negative control group (reconstituted water only) was prepared in parallel. Two replicates with ten daphnids each were exposed to the test item concentrations and the control. A stability control with 1000 mg glyphosate acid/L and no daphnids was also prepared. Immobilisation of *Daphnia* was recorded at 24 and 48 hours after the test initiation. Dissolved oxygen and pH were recorded at the beginning and at the end of the tests. Samples of test media were analysed for glyphosate acid content in freshly prepared and in the aged test media. Glyphosate acid was not detected in the control group. During the 48 hour test period, mean measured concentrations of glyphosate acid were at 86.1% of the nominal concentration. Therefore, reported results are based on nominal glyphosate acid concentrations.

The immobilisation of *Daphnia magna* increased with test concentration. There was a significantly decreasing in test media pH with increasing test concentrations. The drop in pH is considered to have been the likely cause of toxicity. All validity criteria according to the guideline OECD 202 were fulfilled.

In a 48 hour static toxicity test, the 48 hour EC_{50} (immobilisation) for *Daphnia magna* exposed to glyphosate acid was 84.0 mg/L (95% c.i. of 73.3 to 96.6 mg/L). The corresponding 48 hour NOEC (immobilisation) was 60.3 mg glyphosate acid/L.

I. MATERIALS AND METHODS

A. MATERIALS

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1. Test material:

Test item: Glyphosate acid
Description: Solid
Lot/Batch #: 229-Jak-5-1
Purity: 98.9%

2. Vehicle and/or positive control: Reconstituted water (EEC), Potassium dichromate ($K_2Cr_2O_7$)

3. Test organism:

Species: *Daphnia magna*
Age: Neonates (< 24 h old)
Loading: 10 daphnids per 20 mL test medium
Source: [redacted]
Diet/Food: Not fed during test or during the 24 hours preceding test initiation.
Acclimation period: Approximately 24 hours

4. Environmental conditions:

Temperature: 21.0 ± 0.5 °C
Photoperiod: 16 hours light
pH: Control:8.4 – 7.9
62.5 mg test item/L:6.3 – 7.6
125 mg test item/L:4.8 – 5.2
250 mg test item/L:3.2 – 3.4
500 mg test item/L: 2.7 – 2.9
1000 mg test item/L:2.3 – 2.6
Dissolved oxygen: 8.3 – 8.1 mg O_2/L (mean)
Conductivity: Not stated
Hardness: 250 mg $CaC0_3$/L(reconstituted water)

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity test was performed with five test nominal glyphosate acid concentrations of 62.5, 125, 250, 500 and 1000 mg glyphosate acid/L, prepared using reconstituted water (EEC).

The test was conducted using a static test design (without media renewal) over 48 hours, in duplicate 50 mL beakers each containing 20 mL of the appropriate test or control (reconstituted water only) solution. Juvenile Daphnid (<24 hours old) were added impartially to the test vessels until all contained 10 daphnia. In addition, a test item stability control without daphnids was also prepared at 1000 mg glyphosate acid/L.

2. Observations: The number of immobile *Daphnia magna* in each vessel was recorded at 24 h and 48 h after test initiation. The pH-values and oxygen saturation were measured in each test vessel at test initiation and termination. Samples of control and test media were taken at the start – 0 hours (freshly prepared – before animal addition) and end – 48 hours (pooled replicates according to treatment) and analysed for glyphosate content using an HPLC method of analysis.

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3. Statistical calculations: The EC_{50} (immobilisation) was estimated using the Logit-model, NOEC, EC_{50} and EC_{100} values were determined by linear regression.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Measured concentrations of glyphosate acid in the test media at 62.5, 125, 250 and 500 mg glyphosate acid/L were all in the range of 80 – 120% of nominal.

At the highest test concentration of 1000 mg glyphosate acid/L, the concentration at test initiation was 69.7% of nominal and at test termination 85.3%, while in the stability control the concentration was 110.1% and 64.2% of nominal at test initiation and termination, respectively.

The concentrations that bracketed the EC_{50} and NOEC values were in the range between 80 and 120% of nominal. Thus, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The NOEC, EC_{50} and EC_{100} values are given below.

Endpoints	Glyphosate acid [mg/L]
48 h EC_{50}	86.8
48 h EC_{100}	125
48 h EC_{50} (95% CL), Logit-model	84.0 (73.3 – 96.6)
NOEC	60.3

Reference item: The 48h-EC_{50} achieved using the reference item was 1.32 mg potassium dichromate/L (95% CL = 1.203 – 1.426 mg/L), which was within the range of expected toxicity for the reference item. Hence, the sensitivity of this batch of D*aphnia magna* at this test facility was considered acceptable.

B. OBSERVATIONS

The percentage immobilisation increased with increasing test concentration. Beginning with 125 mg test item/L, all daphnids are immobilised after 48 h. The pH of the test solutions significantly decreased with increasing test concentrations, and the drop in pH was considered to be the likely cause of toxicity.

Table 8.3.1.1-2: Effects of glyphosate to *Daphnia magna*

Test parameters	Control	Glyphosate acid [mg/L]									
	-	62.5		125		250		500		1000	
% immobile daphnids after 24 h	0	10	0	30	60	100	100	100	100	100	100
% immobile daphnids after 48 h	0	10	0	100	100	100	100	100	100	100	100
pH after 24 h	8.4	6.3		4.8		3.2		2.7		2.3	
pH after 48 h	7.9	7.6		5.2		3.4		2.9		2.6	

All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

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III. CONCLUSION

The 48 hour EC_{50} (immobilisation) value for *Daphnia magna* exposed to glyphosate acid was 84.0 mg/L with a 95% c.i. of 73.3 to 96.6 mg/L. The 48 hour NOEC value was 60.3 mg glyphosate acid/L based on immobilisation.

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1/03		2000	Acute toxicity of glifosate tecnico Nufarm to *Daphnia magna* Report No: -D51,39/99 Date: 2000-01-17 GLP: yes not published

Guideline: OECD 202 (1984)

Deviations to OECD 202: The concentration of the test substance in the test media was measured only at the beginning of the study. However, glyphosate acid in aquatic studies has been shown to be stable and not measuring the glyphosate concentration at test termination does not affect the validity of this study.

Dates of experimental work: 1999-10-13 to 1999-10-28

Executive Summary

The toxicity of glyphosate acid to *Daphnia magna* was evaluated in a 48-hour static toxicity test conducted with nominal test concentrations of 100, 180, 320, 560, and 1000 mg glyphosate acid/L equivalent to measured concentrations of 103.40, 179.56, 334.11, 597.06, and 1051.12 mg glyphosate acid/L measured at test initiation only. A negative control group (reconstituted water only) was run in parallel. Four replicate vessels each containing 5 Daphnids were prepared for the control group and for each treatment group.

Daphnids in all vessels were observed at 24 and 48 hours for immobilisation. The pH-values and dissolved oxygen concentrations were determined in test media at the start and end of the test. The control and test medium temperatures were measured at the start, at 24 and 48 hours. Glyphosate concentrations were measured only at the start of the test prior to animal addition. Mean measured concentrations ranged from 99.75 to 106.61% of the nominal concentrations. Glyphosate acid was not detected in the control group.

Over 48 hours, there was no immobility of Daphnids up to a mean measured concentration of 179.6 mg a.s./L.. At the 334 mg glyphosate acid/L, there was 10% immobility with 100% immobility at the two highest test concentrations. There was a strong negative correlation between pH value and test item concentrations observed. At 597 mg test item/L, the pH was reduced to 5.0. All validity criteria according to the guideline OECD 202 were fulfilled.

The 48-h EC_{50} (immobilisation) for *Daphnia magna* exposed to glyphosate acid was 420.59 mg glyphosate acid/L based on measured concentration at test initiation. The NOEC after 48 h was 179.56 mg glyphosate acid/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Aspect	White powder
Lot/Batch #:	037-919-113
Purity:	95%

2. Vehicle and/or positive control: Reconstituted water, Potassium dichromate ($K_2Cr_2O_7$)

3. Test organism:

Species:	*Daphnia magna*
Age of animals:	Neonates (< 24 h old)
Loading:	5 organisms per vessel (30 mL glass beakers containing 20 mL test solution)
Supplier:	[redacted] (USA) and maintained as a stock culture at [redacted]

4. Environmental conditions:

Temperature:	20.0 to 21.5°C
pH:	Control(start – 48 h):7.4 – 7.9 100 mg/L(start – 48 h):6.8 – 8.0 180 mg/L(start – 48 h):6.5 – 7.9 320 mg/L(start – 48 h):6.0 – 7.0 560 mg/L (start – 48 h): 5.0 – 5.4 1000 mg/L(start – 48 h):3.1 – 3.4
Dissolved oxygen:	Start of the test: 5.7-6.2 mg O_2/L End of the test: 4.4-4.6 mg O_2/L
Conductivity:	410 mg/L µS/cm
Hardness:	245 mg $CaCO_3$
Photoperiod:	0 hours (exposure phase of test performed in the dark)

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to *Daphnia magna* was evaluated in a 48-hour static toxicity test. Twenty *Daphnia* (comprising 4 replicates of 5 animals per test beaker) were exposed in the negative control group and at each test group. Nominal concentrations were 100, 180, 320, 560, and 1000 mg a.s./L (corresponding to measured concentrations of 103.40, 179.56, 334.11, 597.06, and 1051.12 mg glyphosate acid/L). A reference test using potassium dichromate was performed in parallel to verify the sensitivity of the test system.

2. Observations: Daphnids were observed for immobilisation at 24 and 48 hours and were not fed during the test or during the 24 hour preceding the test. The pH and dissolved oxygen concentrations were determined in the test media at the beginning and at the end of the test. The water temperature in the test media was measured at the start of the test, at 24 and 48 hours. Samples for the determination of the concentrations of glyphosate in the test medium were taken from the control and from all test concentrations at the beginning of the test only.

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3. Statistical calculations: The EC_{50} values for glyphosate and reference substance potassium dichromate were calculated by applying Trimmed Spearman-Karber method.

II. RESULTS AND DISCUSSION

A. FINDINGS

Measured concentrations of glyphosate acid in samples of test media taken at the start of the test ranged between 99.75 and 106.61% of the nominal values. The ecotoxicological endpoints are evaluated based on the initial measured concentrations of the test item.

The NOEC, 24 and 48 hour EC_{50} values (based on measured concentrations) are given in the Table below:

Endpoints	Glyphosate acid [mg a.s./L]
24 h EC_{50}(95% CL)	530.42 (471.64 - 596.52)
48 h EC_{50}(95% CL)	420.59 (388.02 – 455.90)
NOEC	179.56

B. OBSERVATIONS

The immobilisation of *Daphnia magna* during the 48 hour toxicity test is given in Table 8.3.1.1-3.

Table 8.3.1.1-3: Effects of glyphosate acid on *Daphnia magna*

Nominal concentration [mg glyphosate/L]	Measured concentration [mg a.s./L]	Number of exposed *Daphnia* per replicate	Number of immobile *Daphnia* after 24 hours	Immobility after 24 hours [%]	Number of immobile *Daphnia* after 48 hours	Immobility after 48 hours [%]
Control	-	20	0	0	0	0
100	103.40	20	0	0	0	0
180	179.56	20	0	0	0	0
320	334.11	20	0	0	2	10
560	597.06	20	14	70	20	100
1000	1051.12	20	20	100	20	100

The reference substance potassium dichromate resulted in a 48-h EC_{50} of 0.68 mg/L (95% CL = 0.63-0.75 mg/L), which is within the range of historical acceptability.

All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels. The concentration of the test substance in the test media was measured only at the beginning of the study. However, glyphosate acid in aquatic studies has been shown to be stable and not measuring the glyphosate concentration at test termination does not affect the validity of this study.

III. CONCLUSION

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The 48-h EC_{50} (immobilisation) for *Daphnia magna* exposed to glyphosate was 420.59 mg glyphosate acid/L based on initial measured concentrations. The 48 hour NOEC was 179.56 mg glyphosate acid/L.

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./04	.	1996	Glyphosate acid: Acute toxicity to *Daphnia magna* Report No: 5551/B Date: 1996-02-22 GLP: yes not published

Guideline: EPA FIFRA, Subdivision E, Guideline 72-2

Deviations to OECD 202: none

Dates of experimental work: 1995-07-24 to 1995-07-26

Executive Summary

The toxicity of glyphosate acid on *Daphnia magna* was evaluated in a 48-hour static toxicity test. Twenty *Daphnia* (4 replicates of 5 animals per test beaker) per concentration were exposed to nominal 10, 18, 32, 56, 100 and 180 mg glyphosate acid/L and a pH adjusted 1000 mg/L test concentration of glyphosate acid. A negative control group (dilution media only) was run in parallel.

Daphnids were observed for immobilisation at 24 and 48 hours. The pH-values and dissolved oxygen concentrations were determined in the test media at the start (0 hours (before animal addition) and end of the test (48 hours). The temperature in test media was measured at the start 0 hours, at 24 and 48 hours. The concentration of glyphosate acid in the test media was measured at 0 and 48 hours. Glyphosate acid was not detected in the control group. Mean measured concentrations ranged between 85 and 100% of the nominal concentrations. Toxicity was evaluated using mean measured concentrations.

Over the 48 hour test duration, in the control and at measured concentrations up to and including 100 mg a.s./L and also in the pH adjusted 1000 mg glyphosate acid/L treatment group, there was no immobility observed. At 180 mg glyphosate acid/L, there was 100% immobility of *Daphnia magna* after 24 hours.

The immobility result for the pH corrected 1000 mg glyphosate acid/L test group indicated that the toxicity of glyphosate acid to *Daphnia magna* observed at 180 mg glyphosate acid/L was most likely attributable to pH values less than 5. All validity criteria according to the guideline OECD 202 were fulfilled.

The 48-h EC_{50} (immobilisation) for *Daphnia magna* exposed to glyphosate acid was 130 mg glyphosate acid/L. The corresponding 48 hour NOEC value was 100 mg glyphosate acid/L.

The pH adjusted 48-h EC_{50} for *Daphnia magna* exposed to glyphosate acid was considered to be >1000 mg glyphosate acid/L, with a corresponding 48 hour NOEC of 1000 mg glyphosate acid/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Aspect	White solid
Lot/Batch #:	P24
Purity:	95.6%

2. Vehicle and/or positive control: Elendt M4

3. Test organism:

Species:	*Daphnia magna* Straus
Age of animals:	Neonates (< 24 h old)
Loading:	5 organisms per vessel (250 mL glass beakers containing 200 mL test solution) which corresponds to 25 *Daphnia*/L.
Source:	

4. Environmental conditions:

Temperature:	20.5-20.8°C
pH:	Control(start – 48 h):8.2 – 8.1 10 mg/L(start – 48 h):7.7 – 8.0 18 mg/L(start – 48 h):7.4 – 8.0 32 mg/L(start – 48 h):7.0 – 7.8 56 mg/L (start – 48 h): 6.6 – 7.5 100 mg/L(start – 48 h):5.7 – 6.1 180 mg/L(start – 48 h):4.3 – 4.2 1000 mg/L(pH, adjusted, start – 48 h):9.0 – 8.8
Dissolved oxygen:	8.7-9.0 mg O_2/L
Conductivity:	693 mg/L µS/cm
Hardness:	263 mg $CaCO_3$
Photoperiod:	16 hours light with a 20 minute transition period

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid on *Daphnia magna* was evaluated in a 48-hour static toxicity test. Twenty *Daphnia* (4 replicates of 5 animals per test beaker) per concentration were exposed to nominal 10, 18, 32, 56, 100 and 180 mg/L of glyphosate acid and a pH adjusted 1000 mg/L test concentration of glyphosate acid. In addition, 4 x 5 *Daphnia* were exposed in a negative control group (dilution medium only).

A stock solution was prepared at a nominal concentration of 1000 mg glyphosate acid/L by dissolving 1000 mg of glyphosate acid in 1000 mL dilution water. The 10 to 180 mg glyphosate acid test media were prepared by diluting aliquots of the stock solution with dilution water.

A further 1000 mg a.s./L stock solution was prepared by dissolving 1000 mg of glyphosate acid in 1000 mL of dilution water. The pH of this stock solution was adjusted from 2.59 to 8.98 using 12 mL of 1 molar

sodium hydroxide. All stock and test mediums were observed to be clear and colourless. The *Daphnia* were randomly placed into the test beakers and exposed to the test item for 48 hours.

2. Observations: Daphnids were observed for immobilisation at 24 and 48 hours and were not fed during the test nor during the 24 hours period prior to test initiation. The pH-values and dissolved oxygen concentrations were determined in the test media at the start and end of the test. The temperature of the test media was measured at the start of the test, at 24, and 48 hours. The concentrations of glyphosate acid in samples of test media were measured at 0 and 48 hours.

3. Statistical calculations: The LC_{50} values and their 95% confidence intervals were calculated using non-linear interpolation. The NOEC was determined by visual interpretation of the data..

II. RESULTS AND DISCUSSION

A: FINDINGS

The measured concentrations of glyphosate acid in samples of test media taken at the start and end of the test ranged between 85 and 100% of the nominal values. Therefore, the ecotoxicological endpoint results are based on nominal glyphosate acid concentrations..

Due to an oversight at 0 hours the pH adjusted 1000 mg a.s./L test solution was not sampled for analysis and therefore a sample was taken at 24 hours. The lack of 0 hour analysis for this concentration was considered not to have affected the validity of the study since analysis at 24 and 48 hours gave results which were close to the nominal value (100 and 83%, respectively).

The endpoints for glyphosate acid exposure to *Daphnia magna* are given in the table below.

Endpoints	Glyphosate acid [mg a.s./L]	Glyphosate acid (pH-adjusted) [mg a.s./L]
24 h EC_{50}	130 (100-180)	>1000
48 h EC_{50}	130 (100-180)	>1000

B. OBSERVATIONS

The 24 and 48 hour EC_{50} values (based on nominal concentrations of glyphosate acid) are given in Table 8.3.1.1-4.

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Table 8.3.1.1-4: Effects of glyphosate acid on *Daphnia magna*

Nominal concentration [mg a.s./L]	Number of exposed *Daphnia* per replicate	Number of immobile *Daphnia* after 24 hours	Immobility after 24 hours [%]	Number of immobile *Daphnia* after 48 hours	Immobility after 48 hours [%]
Control	20	0	0	0	0
10	20	0	0	0	0
18	20	0	0	0	0
32	20	0	0	0	0
56	20	0	0	0	0
100	20	0	0	0	0
180	20	20	100	20	100
1000 (pH adjusted)	20	0	0	0	0

The results obtained from this study indicate that the toxicity of glyphosate acid below 1000 mg/L was caused by pH values less than 5.

All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

III. CONCLUSION

The 48-h EC_{50} (immobilisation) for *Daphnia magna* exposed to glyphosate acid was 130 mg glyphosate acid/L. The corresponding 48 hour NOEC value was 100 mg glyphosate acid/L.

The pH adjusted 48-h EC_{50} for *Daphnia magna* exposed to glyphosate acid was considered to be >1000 mg glyphosate acid/L, with a corresponding 48 hour NOEC of 1000 mg glyphosate acid/L.

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Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./05	[redacted]	1994	Acute Toxicity in *Daphnia magna*; Test Article: 'Glyphosate isopropylamine salt' [redacted] Report No: 83-91-0737-00-93 Date: 1994-01-28 GLP: yes not published

Guideline: OECD Guideline 202; ECC directive 92/69

Deviations to OECD 202: none

Dates of experimental work: January 04, 1994 – January 06, 1994

Executive Summary

The acute toxicity of glyphosate isopropylamine salt on *Daphnia magna* was evaluated in a 48-hour static toxicity test. The test was performed using static conditions as limit test using only one test concentration of nominally 100 mg test item/L, equivalent to 61.6 mg glyphosate isopropylamine salt/L or 45.64 mg glyphosate acid/L. In addition, a negative control group (Elendt M4 only) was run in parallel (Elendt-medium). There were four replicate vessels in the control and per treatment group, each containing five

Daphnia magna (25 mL volumetric cylinder containing 10 mL test medium). Temperature, pH-value and oxygen saturation of the test solutions were measured at test initiation and termination. The number of immobile daphnids in all vessels was recorded 24 and 48 h after test initiation. Samples for the determination of the concentrations of glyphosate in the test medium were taken at the start (before animal addition) and end of the test. Glyphosate acid was not detected in the control group. The mean measured analysed test concentration was 103% of the nominal value. Therefore, the results reported are related to nominal concentrations of the test item.

At 100 mg test item/L, there were no immobilised *Daphnia magna* after 48 hours exposure to glyphosate isopropylamine salt. All validity criteria according to OECD 202 were fulfilled.

The acute toxicity of glyphosate isopropylamine salt exposed to *Daphnia magna* was determined in a 48 hour static toxicity test. The 48 hour EC_{50} (immobilisation) was >100 mg test item/L, equivalent to 61.6 mg glyphosate isopropylamine salt/L or 45.64 mg glyphosate acid/L (nominal).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine salt
Description:	viscous liquid
Lot/Batch #:	01/06/93
Purity:	61.6% Glyphosate isopropylamine salt; 456.43 mg a.e./L
Density:	1.23 g/cm^3 at 20°C

2. Vehicle and/or positive control: Elendt M4, 0.4 and 1.4 mg/L $K_2Cr_2O_7$

3. Test organism:

Species:	*Daphnia magna* Strauss
Age:	neonates (6 - 24 h old)
Loading:	10 mL for 5 specimens
Source:	[redacted]
Diet/Food:	None
Acclimation period:	Daphnids were held in groups of 25-30 organisms in 1000 mL glass vessels at test conditions. Specimens were fed on green algae and water was renewed 3 times a week.

4. Environmental conditions:

Temperature:	20.5°C
Photoperiod:	16 hours Light intensity = 600 – 700 lux
pH:	7.41 – 7.66
Dissolved oxygen:	> 60% of air saturation 8.0 – 8.3 mg O_2/L
Conductivity:	0.049 µS/cm
Hardness:	14.5° dH

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: The acute toxicity test was performed under static conditions as a limit test at a nominal test concentration of 100 mg test item/L, equivalent to 61.6 mg glyphosate isopropylamine salt/L or 45.64 mg glyphosate/L in glass volumetric cylinders containing reconstituted water (Elendt-M4). In addition a negative control group (Elendt-M4) was prepared in parallel.

Two reference toxicant groups were exposed to 0.4 and 1.4 mg/L of $K_2Cr_2O_7$, with four vessels per treatment, each containing five *Daphnia magna* (25 mL volumetric cylinder containing 10 mL test medium).

2. Observations: All *Daphnia magna* were observed after 24 and 48 hours after initiation of the test. Temperature, pH-value and oxygen saturation of the test solutions were measured at initiation and test termination.

Total number of immobile *Daphnia magna* was recorded at 24 h and 48 h after the test initiation.

Analytical measurement of the test item concentration was performed using an HPLC method of analysis at the start (0 h) and end (48h) of the limit test. Glyphosate isopropylamine salt concentrations were determined based on measured concentrations of glyphosate acid.

3. Statistical calculations: Since the immobility was < 50%, no statistical calculation of EC_{50} values was possible.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: The measured concentrations of glyphosate acid in test media at the start (0h) and end (48h) of the limit test were 47.322 mg glyphosate acid/L (103.7%) and 47.091 mg glyphosate acid/L (103.2%) respectively. As measured concentrations ranged between 80 and 120% of nominal concentration, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The EC_{50} and NOEC values for Glyphosate isopropylamine salt exposure to *Daphnia magna* are given below.

Endpoints	test item [mg/L]	Glyphosate isopropylamine salt [mg/L]	Glyphosate Acid [mg a.s/L]
EC_{50} (48 h)	> 100	> 61.6	> 45.64
NOEC	100	61.6	45.64

B. OBSERVATIONS

Observations: The immobility rate in the control group did not exceed 10% (0% in the test) at any stage of the test. At the concentration level of 100 mg test item/L, none of the daphnids tested were found to be immobilised, 24 h and 48 h after the start of the test.

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Table 8.3.1.1-5: Immobilisation of daphnids exposed to glyphosate isopropylamine salt

	Control	Test item [mg/L]	Toxic reference item [mg/L]	
test item	**-**	**100**		
glyphosate isopropylamine salt	**-**	**61.6**	**0.4**	**1.4**
glyphosate	**-**	**45.64**	**0.4**	**1.4**
% immobilisation after 24 h	0	0	0	85
% immobilisation after 48 h	0	0	5	100

The 48 h EC_{50} obtained for the reference substance was within the range of 0.4 to 1.4 mg/L, indicating the integrity of the test system. All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥3 mg/L in all test vessels.

III. CONCLUSION

In a 48-hours static acute toxicity study with *Daphnia magna*, the EC_{50} was determined to be>100 mg test item/L, equivalent to 61.6 mg glyphosate isopropylamine salt/L or 45.64 mg glyphosate acid/L (nominal).

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./06		2000	Acute toxicity of glifosato IPA tecnico Nufarm to *Daphnia magna* Report No: -D51.017/00 Date: 2000-07-31 GLP: yes not published

Guideline: OECD 202 (1984)

Deviations to OECD 202: The concentration of the test substance in the test media was measured only at the beginning of the study. However, glyphosate in aquatic studies has been shown to be stable and not measuring the glyphosate concentration at test termination does not affect the validity of this study.

Dates of experimental work: 2000-06-06 to 2000-06-15

Executive Summary

The toxicity of glyphosate isopropylamine salt on *Daphnia magna* was evaluated in a 48-hour static toxicity test. Twenty *Daphnia* (4 replicates of 5 animals per test beaker) per concentration were exposed to 75.9, 150.0, 282.8, 693.6 and 1397 mg/L effective concentrations, equivalent to 25.6, 50.5, 95.4, 233.3 and 470.9 mg a.e./L. In addition, 4 x 5 *Daphnia magna* were exposed in a negative control group (Elendt M4 only) that was run in parallel. All Daphnids were observed for immobilisation after 24 and 48 hours exposure. The pH-values and dissolved oxygen concentrations were measured in the test media at the start and end of the test. The temperature in the test media was measured at the start of the test, at 24, and 48 hours. Samples for the determination of the concentrations of glyphosate in the test medium were taken from the control and from all test concentrations at the start of the test (before animal addition). Glyphosate isopropylamine salt was not detected in the control group. The measured test concentrations

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ranged between 75.90 and 139.70% of the nominal values. Therefore, the ecotoxicological endpoints are evaluated using measured concentrations of the test item.

At 48 hours, there were no immobilised *Daphnia magna* observed at any of the test concentrations. All validity criteria according to the guideline OECD 202 were fulfilled.

The 48-h EC_{50} for *Daphnia magna* exposed to glyphosate isopropylamine salt was greater than 470.9 mg a.e./L based on measured concentration. The NOEC after 48 h based on immobilisation was 470.9 mg a.e./L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine salt
Lot/Batch #:	MJRT 025-201-104
Purity:	612.7 g/kg salt equivalent, 470.9 g a.e./L

2. Vehicle and/or positive control: Reconstituted water, Toxic standard (potassium dichromate)

3. Test organism:

Species:	*Daphnia magna*
Age of animals:	Neonates (< 24 h old)
Loading:	5 organisms per vessel (30 mL glass beakers containing 20 mL test solution)
Source:	[redacted] [redacted] (USA) and maintained as a stock culture at [redacted]

4. Environmental conditions:

Temperature:	21.1 to 21.2 C°
pH:	Start of the test: 5.56-7.39 End of the test: 5.54-7.81
Dissolved oxygen:	Start of the test: 6.10-6.27 mg O_2/L End of the test: 5.57-5.67 mg O_2/L
Conductivity:	603.0 mg/L µS/cm
Hardness:	248 mg $CaCO_3$
Photoperiod:	0 hours (exposure phase performed in the dark).

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate isopropylamine salt to *Daphnia magna* was evaluated in a 48-hour static toxicity test. Twenty *Daphnia* (4 replicates of 5 animals per test beaker) per concentration were exposed to 100, 180, 320, 560, and 1000 mg a.s./L nominal concentrations, equivalent to 75.9, 150.0, 282.8, 693.6 and 1397 mg/L effective concentrations, (25.6, 50.5, 95.4, 233.3 and 470.9 mg a.s./L). In addition, 4 x 5 *Daphnia* were exposed to test water without test substance (negative control). A reference test using potassium dichromate was run in parallel to verify the sensitivity of the test system. A

primary stock solution at a nominal concentration of 1000 mg a.s./L was prepared by dissolving 500 mg test item in 500 mL reconstituted water. Appropriate amounts of the primary stock were diluted to prepare the lower test concentrations of 100, 180, 320, and 560 mg test item/L. The *Daphnia* were randomly placed into the test beaker and exposed to the test item for 48 hours.

2. Observations: Daphnids were observed for immobilisation at 24 and 48 hours and were not fed during the test or during the 24 hour period preceding test initiation. The pH-values and dissolved oxygen concentrations were determined in the test media at the start and at the end of the test. The temperature of the test media was measured at the start of the test, at 24 and 48 hours. Samples for the determination of the concentrations of glyphosate in the test medium were taken from the control and from all test concentrations at the start of the test (before animal addition).

3. Statistical calculations: Since the immobility was < 50% at the highest concentration tested, no statistical calculation of EC_{50} values was possible.

II. RESULTS AND DISCUSSION

A: FINDINGS

The measured concentrations of the test item in samples of test media taken at the start of the test ranged between 75.90 and 139.70% of the nominal values. Therefore, the ecotoxicological endpoints are evaluated using measured concentrations of the test item.

The endpoints for *Daphnia magna* exposure to glyphosate isopropylamine salt are given in the table below.

Endpoints	Glyphosate IPA salt [mg a.s./L]	Glyphosate acid [mg a.s./L]
48 h EC_{50}	> 1397	>470.9
NOEC	1397	470.9

B. OBSERVATIONS

The effects of glyphosate IPA salt on *Daphnia magna* immobilisation are given in Table 8.3.1.1-6.

Table 8.3.1.1-6: Effects of glyphosate IPA salt on *Daphnia magna*

Nominal Glyphosate IPA salt concentration [mg glyphosate/L]	Measured concentration [mg glyphosate acid/L]	Number of exposed *Daphnia* per replicate	Number of immobile *Daphnia* after 24 hours	Number of immobile *Daphnia* after 48 hours
Control	-	20	0	0
100	25.6	20	0	0
180	50.5	20	0	0
320	95.4	20	0	0
560	233.3	20	0	0
1000	470.9	20	0	0

The reference substance potassium dichromate resulted in a 48-h EC_{50} (immobilisation) of 1.22 mg/L (95% CL = 1.12-1.35 mg/L).

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After 24 and 48 hours of exposure, there was no immobilisation of *Daphnia magna* observed in the control or in the test item concentration vessels.

All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

III. CONCLUSION

The 48-h EC_{50} for *Daphnia magna* exposed to glyphosate isopropylamine salt was greater than 470.9 mg a.s./L based on measured concentration. The NOEC after 48 h based on immobilisation was 470.9 mg a.s./L.

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./07	[redacted]	2003	MON 78623: A 48-HOUR STATIC ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*) [redacted] [redacted] USA Report No [redacted]-2002-150 Date: 2003-05-01 GLP: yes not published

Guideline: OECD Guideline 202 (1984)
OPPTS 850.1010 (1996)
EU Method C2 (1992)

Deviations to OECD 202: Additionally, immobilisation was recorded after 19 h of exposure.

Dates of experimental work: 2003-12-03 to 2003-12-05

Executive Summary

The effects of Glyphosate K-salt on *Daphnia magna* were evaluated in a 48-hour static toxicity test performed using nominal concentrations of 156, 313, 625, 1250 and 2500 mg test item/L (equivalent to mean measured concentrations of 165, 312, 624, 1285 and 2582 mg test item/L). In addition, a negative control group (well water only) was run in parallel. There were two vessels prepared for the control and for each treatment, each containing ten daphnids.

The total number of immobile *Daphnia magna* was recorded at 19 h, 24 h and 48 h after test initiation. Mean measured concentrations were recorded at the beginning and at the end of the tests.

The analysed test concentrations ranged between 97 and 107% of the nominal values. Glyphosate K-salt was not detected in the control group. At 624 mg test item/L 65% of the daphnids were observed to be lethargic at the bottom of the test chamber at test termination. Immobility at 48 h at concentrations of 1285 and 2582 mg test item/L were5 and 25%, respectively and all remaining daphnids at these two test concentrations were lethargic at the bottom of the test chamber. All validity criteria according to the guideline OECD 202 were fulfilled.

In conclusion, the 48 h EC_{50} for *Daphnia magna* exposed to Glyphosate K-salt was calculated to be > 2582 mg/L, equivalent to >1231.6 mg glyphosate acid/L based on mean measured concentrations. The 48- hour no-effect level (NOEC) for Glyphosate K-salt was determined to be 312 mg/L, equivalent to 148.8 mg glyphosate acid/L based on mean measured concentrations.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 78623 (Glyphosate K-salt)
Active substance	Glyphosate acid
Description:	Yellow liquid
Lot/Batch #:	GLP-0108-11688-F
Purity:	47.7%
2. Vehicle and/or positive control:	Well water
3. Test organism:	
Species:	*Daphnia magna*
Age:	Neonates (< 24 h old)
Loading:	2 × 10 specimens for 250 mL test solution
Source:	[redacted]
Diet/Food:	None
Acclimation period:	None
4. Environmental conditions:	
Temperature:	19.5 – 20.0°C
Photoperiod:	16 hours light / 8 hours dark with 30 min transition period
pH:	5.7 – 8.1 (test item) 8.1 – 8.2 (control)
Dissolved oxygen:	≥ 8.6 mg/L (≥ 96% saturation)
Conductivity:	310 µmhos/cm
Hardness:	140 mg $CaCO_3$/L.
Alkalinity:	184 mg $CaCO_3$/L

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finding test, a definitive toxicity test was performed using nominal concentrations of 156, 313, 625, 1250 and 2500 mg test item/L (mean 165, 312, 624, 1285 and 2582 mg test item/L in a static test setup. The test solutions were prepared using test facility well water (Dissolved oxygen ≥ 96%, pH = 5.7 – 8.1, hardness 140 mg $CaCO_3$/L.). In addition, a control group was exposed to well water (negative control). There were two replicates per treatment, each containing ten daphnids. Test chambers were 250 mL glass beakers containing approx. 250 mL of test medium.

2. Observations: Total number of immobile *Daphnia magna* was recorded at 19h, 24 h and 48 h after the test initiation. Temperature of the test solutions was measured at the test initiation and termination. Hardness, alkalinity and specific conductance of the dilution water were measured at test initiation. The pH value and oxygen saturation were measured at test initiation and at 24h and 48h. For analysis of test

substance concentration with HPLC, test medium was collected from the replicate test chambers at 0 and 48 h.

3. Statistical calculations: Since the immobility was < 50%, no statistical calculation of EC_{50} values was possible. Therefore, EC_{50} and NOEC values were determined by visual inspection.

II. RESULTS AND DISCUSSION

A. FINDINGS

The analytics confirm the stability of the test substance, since the recovery was 99 – 105% at test start and 97 – 107% at test end. Results are based on mean measured concentrations.

The EC_{50} and NOEC are based on mean measured concentrations of 165, 312, 624, 1285 and 2582 mg test item/L and are given below.

Endpoints	Glyphosate K-salt [mg/L]	Glyphosate Acid [mg/L]
48 h EC_{50}	> 2582	> 1231.6
NOEC	312	148.8

B. OBSERVATIONS

In the negative control and at mean measured concentrations of 165 and 312 mg test item/L no effects were observed. At 624 mg test item/L 65% of the daphnids were observed to be lethargic at the bottom of the test chamber at test termination. Immobility at 48 h at 1285 and 2582 mg test item/L was 5 and 25%, respectively. All remaining daphnids were lethargic at the bottom of the test chamber.

Table 8.3.1.1-7: Lethal effects of glyphosate K-salt to *Daphnia magna*

Mean measured **Glyphosate K-salt (MON 78623) [mg/L]**	**Control**	**165**	**312**	**624**	**1285**	**2582**
Mean Measured Glyphosate acid [mg/L]	**-**	**78.7**	**148.8**	**297.6**	**612.9**	**1231.6**
Immobility (19 h) [%]	0	0	0	0	0	0
Immobility (24 h) [%]	0	0	0	0	0 (8C)	0 (17C)
Immobility (48 h) [%]	0	0	0	0 (13C+G)	1 (19C+G)	5 (15C+G)

C = lethargic ; G = on bottom of test chamber; AN = appear normal

All validity criteria according to OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

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III. CONCLUSION

In conclusion, the 48 h EC_{50} for *Daphnia magna* exposed to Glyphosate K-salt was calculated to be > 2582 mg/L, equivalent to >1231.6 mg glyphosate acid/L based on mean measured concentrations. The 48- hour no-effect level (NOEC) for Glyphosate K-salt was determined to be 312 mg/L, equivalent to 148.8 mg glyphosate acid/L based on mean measured concentrations.

Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./08	[redacted]	1998	Acute Toxicity Study in *Daphnia magna* with (Aminomethyl)Phosphonic Acid (Static). [redacted] Report No: 232471 Date: 1998-06-29 GLP: yes Not published

Guideline:	OECD Guideline 202, Part I (1984) ECC Directive 92/69, Part C.2 (1992) ISO International Standard 6341 (1996)
Deviations:	None
Dates of experimental work:	1998-05-18 to 1998-05-27

Executive Summary

The toxicity of Aminomethyl-phosphonic acid (AMPA) on *Daphnia magna* were evaluated in a 48-hour static toxicity test conducted as a limit test at a nominal concentration of 100 mg AMPA/L. A negative control group (Elendt M4 only) was run in parallel. Twenty daphnids (2 replicates each containing 10 individuals per replicate) were exposed in the control and at each treatment level.

Immobilisation was recorded 24 and 48 hours after the start of the test. The concentration of AMPA in the test solutions were measured at 0 and 48 hours.

AMPA was not detected in the control group. The mean measured test concentrations of AMPA ranged from 95 to 98% of the nominal values, therefore, the ecotoxicological endpoints were evaluated using the nominal concentrations of the test item.

At the tested nominal concentration of 100 mg AMPA/L, no immobilisation was observed in the control and across treatment groups during the 48 h exposure time. All validity criteria according to OECD 202 were fulfilled.

The 48 hour EC_{50} for *Daphnia magna* exposed to AMPA in a limit test at 100 mg AMPA/L was determined to be > 100 mg AMPA/L

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Aminomethylphosphonic acid (AMPA)
Description:	White powder

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Lot/Batch #: A010047101
Purity: 99%

2. Vehicle and/or positive control: Elendt M4; Reference item: $K_2Cr_2O_7$

3. Test organism:

Species: *Daphnia magna* Straus
Age: Neonates (< 24 h old)
Loading: 10 daphnids per 80 mL of test medium
Source: [redacted]

4. Environmental conditions:

Temperature: 20.4 – 20.6°C
Photoperiod: 16 hours light
pH: 8.0 – 8.2 (control), 6.2 - 6.4 (test solution)
Dissolved oxygen: 8.8 – 9.0 mg O_2/L
Hardness: 250 mg $CaC0_3$/L

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range finding test, a 48 hour limit toxicity test performed at a nominal limit concentration of 100 mg AMPA/L, prepared using ISO-medium. A negative control group (ISO-media only) was run in parallel. Two replicates were prepared for the control and per treatment group comprising 100 mL vessels each containing 80 mL of the appropriate test solution with each replicate containing 10 daphnids.

2. Observations: The total number of mobile *Daphnia magna* was recorded at 24 h and 48 h after test initiation. The pH-values and oxygen saturation of the test solutions were measured at test initiation and termination. The temperature was measured daily in one control vessel. The concentrations of AMPA in samples of control and test media were taken (from preparation flasks) at the start of the test and from pooled replicate according to treatment at the end of the test (48 hours).

3. Statistical calculations: Since the immobility was < 50%, no statistical calculation of EC_{50} values was possible.

II. RESULTS AND DISCUSSION

A. FINDINGS

The $EC_{50}$and NOEC value is given below based on nominal concentrations.

Endpoints	AMPA[mg/L]
48 hour EC_{50}	> 100
48 hour NOEC	100

Analytical data: Measured concentrations of AMPA in test media at the start of the test were 98% of AMPA was recovered. In the aged test media measured concentrations were 95% of the nominal concentration. As the mean measured concentrations of AMPA were between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal AMPA concentrations of the test item.

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Reference test: The 48h-EC_{50} for the reference item was 0.5 mg/L (95% CL = 0.4 – 0.6 mg/L), which was within the range of expected responses. The sensitivity of this batch of D*aphnia magna* was in agreement with the historical data collected at test facility.

B. OBSERVATIONS

At the tested nominal concentration, there was no immobilisation of daphnids observed during the 48 h exposure time. All validity criteria according to OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

III. CONCLUSION

The 48 hour EC_{50} for *Daphnia magna* exposure to AMPA determined in a 48 hour limit test was determined to be > 100 mg AMPA/L. The 48 hour NOEC was considered to be 100 mg AMPA/L.

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Annex point	Author(s)	Year	Study title
IIA 8.3.1.1./09	[redacted]	2011	HMPA (Hydroxymethylphosphonic acid): A 48-hour static acute toxicity test with the cladoceran (*Daphnia magna*) [redacted] USA Report No: [redacted]-2010-329 Date: 2011-03-29 GLP: yes Not published

Guideline: OECD 202 (1984)
EPA OPPTS 850.1010

Deviations to OECD 202: None

Dates of experimental work: 2011-01-25 to 2011-01-28

Executive Summary

The toxicity of Hydroxymethylphosphonic acid (HMPA) on *Daphnia magna* was evaluated in a 48-hour static toxicity test. *Daphnia magna* neonates were exposed to a limit concentration of 100 mg HMPA/L and a negative control consisting of dilution water only. The test consisted of three replicates per treatment group and control with 10 daphnids exposed per replicate vessel. *Daphnia* were not fed during the test. All Daphnids were observed for immobilisation and other clinical signs of toxicity at 2.5, 24 and 48 hours after test initiation.

Temperature, pH-values and dissolved oxygen concentrations were measured at the beginning, at approximately 24 hours during the test and at the end of the test. Samples control and the test item treatment media were taken and analysed for HMPA concentration at the beginning of the test and at 48 hours from each replicate test chamber. HMPA was not detected in the control group. The measured test concentrations ranged between 86 and 103% of the nominal values.

There was no immobility or overt signs of toxicity observed in the treatment group or in the control. All validity criteria according to the OECD guideline 202 were fulfilled.

The 48-hour EC_{50} for *Daphnia magna* exposed to HMPA was > 100 mg HMPA/L. The 48- hour NOEC was determined to be 100 mg HMPA/L

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: HMPA(Hydroxymethylphosphonic acid)
Description: White powder
Lot/Batch #: GLP-1003-20448-A
Purity: 97.0%

2. Vehicle and/or positive control: Well water

3. Test organism:

Species: *Daphnia magna* Straus
Age: Neonates (< 24 h old)
Loading: 10 daphnids per 220 mL of test medium
Source: [redacted]
Diet/Food: None
Acclimation period: None

4. Environmental conditions:

Temperature: 19.7 – 20.7 °C
Photoperiod: 16 hours light (light intensity = 323 Lux), with 30 minute transition periods.
pH: 6.9 – 8.5
Dissolved oxygen: 8.3 – 9.4 mg O_2/L (≥92% of O_2 saturation)
Conductivity: 386 µS/cm
Hardness: 140 mg $CaC0_3$/L

B. STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of Hydroxymethylphosphonic acid (HMPA) on neonates of *Daphnia magna* was evaluated in a 48-hour static toxicity test at a single nominal limit concentration of 100 mg HMPA/L dissolved in well water. A negative control group (well water only) was prepared in parallel. Thirty daphnids (3 replicates of 10 animals per test beaker) were exposed at the control and at the limit concentration.

2. Observations: The total number of immobile *Daphnia magna* was recorded at 2.5, 24 h and 48 h after test initiation. In addition, specimens were observed for clinical signs of toxicity.

Temperature, pH-values and oxygen saturation of the test solutions were measured at test initiation, after 24 hours and at test termination (48 h). The temperature of test media was monitored continuously in all test vessels. Hardness, alkalinity, specific conductance and total organic carbon (TOC) were measured at the beginning of the test.

Samples of test media were taken from each replicate test chamber at the start and end of the test for the determination of HMPA concentrations. Samples were analysed using an HPLC method of analysis with mass selective detection (LC/MS).

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3. Statistical calculations: Descriptive only since no immobility of daphnids was observed in the test and control treatments.

II. RESULTS AND DISCUSSION

A. FINDINGS

The measured test concentrations ranged between 86 and 103% of the nominal values. Therefore, the EC_{50} and NOEC values given below are based on nominal concentrations.

Endpoints	HMPA [mg/L]
48 hour EC_{50}	>100 mg/L (nominal)
48 hour NOEC	100 mg/L (nominal)

B. OBSERVATIONS

After 2.5, 24 and 48 hours of exposure, no immobilisation of *Daphnia* in the control nor in the test item concentration vessels was observed.

All validity criteria according to the OECD 202 were fulfilled, as no immobility of daphnids was observed in control groups and dissolved oxygen concentration was ≥ 3 mg/L in all test vessels.

III. CONCLUSION

The 48-hour EC_{50} for *Daphnia magna* exposed to HMPA was >100 mg a.s./L. The 48- hour NOEC was determined to be 100 mg HMPA/L.

IIA 8.3.1.2 Acute toxicity (24 and 48 hour) for representative species of aquatic insects

As glyphosate acid is not an insecticide or insect growth regulator, studies on the acute toxicity for aquatic insects are not required.

IIA 8.3.1.3 Acute toxicity (24 and 48 hour) for representative species of aquatic crustaceans (species unrelated to Daphnia)

As glyphosate acid is not an insecticide or insect growth regulator, studies on the acute toxicity for representative species of aquatic crustaceans (species unrelated to Daphnia) are not required.

IIA 8.3.1.4 Acute toxicity (24 and 48 hour) for representative species of aquatic gastropod molluscs

This is not a data requirement according to Regulation 1107/2009/EC.

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IIA 8.3.2 Chronic toxicity to aquatic invertebrates

As summarized in the 2001 EU evaluation, glyphosate acid has low chronic toxicity to aquatic vertebrates. Chronic NOEC values >1 mg/L for aquatic invertebrates is categorized by Canton et al. (1991)[4] as practically nontoxic. The lowest chronic NOEC for a *Daphnia* life-cycle study was 30 mg a.s./L. Additionally, a *Daphnia* life-cycle study was performed for AMPA that also demonstrates low chronic toxicity with a NOEC of 15 mg/L. A summary of data for chronic toxicity of aquatic invertebrates reviewed in the 2001 EU glyphosate evaluation and the new study performed for AMPA is included in Table 8.3.2-1.

Table 8.3.2-1: Chronic toxicity of glyphosate acid, glyphosate IPA salt and its metabolite AMPA to aquatic invertebrates

Species	Test design	EC_{50} (mg a.s./L)	NOEC (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid					
Daphnia magna	21 d semi-static		50	IIA 8.3.2.1/01 [redacted] 6535/B [redacted] 1999/yes	-
Daphnia magna	21 d semi-static	>100	52	141874 [redacted] 1995/yes	95-00065
Daphnia magna	21 d flow-through		50	[redacted] 82-036 [redacted] 1982/no	95-00010
Daphnia magna	21 d semi-static	>100	100	[redacted] 89-58 [redacted] 1989/no	95-00008
Daphnia magna	21 d semi-static		**30 mg/L**	250795 [redacted] 1990/no	95-00733
Daphnia magna	21 d	-	95	-	94-00154 [redacted] 1990/no
Daphnia magna	21 d	-	56	-	96-00066 [redacted] 1995/no
Glyphosate-IPA salt					
Daphnia magna	21 d semi-static	-	57.9	89-91-2328-05-93 [redacted] 1993/yes	95-00549
AMPA					
Daphnia magna	21 d semi-static	90	15	IIA 8.3.2.1/02 [redacted]-2010-327 [redacted] [redacted]./yes	-

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

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For chronic toxicity of daphnids exposed to glyphosate-IPA salt, in SANCO/6511/VI/99-final a NOEC of 455 mg/L was proposed. However, due to the fact that a significant reduction of reproduction was observed at 207 and 455 mg test item/L, the NOEC for reproduction was re-determined to be 94 mg test item/L, equivalent to 57.90 mg glyphosate isopropylamine salt/L and 42.90 mg glyphosate acid/L (nominal) (see [redacted] 1993, IIA 8.2.5/02).

[4] Canton et al. 1991. Catch-up operation on old pesticides. National Institute of Public Health and Environmental Protection, Bilthoven. Report no. 678801001.

IIA 8.3.2.1 Chronic toxicity in *Daphnia magna* (21-day)

Annex point	Author(s)	Year	Study title
IIA 8.3.2.1/01	[redacted] .	1999	Glyphosate acid: Chronic toxicity to *Daphnia magna* [redacted] Report No: [redacted]6535/B Date: 1999-06-29 GLP: yes Published

Guideline: OECD 202, Part II, Reproduction Test (1984)

Deviations to OECD 211: None.

Dates of experimental work: 1998-11-16 to 1998-12-07

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Executive Summary

The lethal and sub lethal effects of glyphosate acid on *Daphnia magna* were evaluated in a 21-day toxicity test performed under semi-static conditions. Ten replicates of one *Daphnia* per concentration were exposed to 12.5, 25, 50, 100, and 200 mg a.s./L nominal concentrations. In addition, 10 x 1 *Daphnia* were exposed to test medium without test substance (blank control). The *Daphnia* were randomly placed into the test beaker and exposed to the test item for 21 days. The test *Daphnia* were fed daily with cultured algae (*Chlorella vulgaris*).

Mortality of P_0 generation of *Daphnia* and observation for the presence of alive and dead offspring (termed F_1 generation) were recorded daily in each test vessel. At the end of the test, the length of each surviving P_0*Daphnia* was measured.

The pH was measured in each newly prepared test solution. The pH and dissolved oxygen concentration of two of the replicates of the old test solutions were measured after transfer of the P_0 generation of daphnids. Temperature measurements were recorded daily by means of a thermometer and hourly automatically. The concentration of glyphosate acid in the test solutions was determined on days 0, 2, 7, 9, 14, and 16. Old solutions were analysed on days 2, 7, 9, 14, and 21.

Glyphosate acid was not detected in the control group. The mean measured concentrations of glyphosate acid in the new test solutions ranged from 100 to 104% of the nominal values. The mean measured concentrations in the old test solutions ranged from 96 to 104% of the nominal values. Therefore, the results are based on nominal glyphosate acid concentrations. The overall 21-day NOEC for the reproduction of *Daphnia magna* exposed to glyphosate acid was 50 mg/L based on nominal concentration. All validity criteria according to the pertinent OECD 211 guideline were fulfilled.

The overall 21-day NOEC for the reproduction of *Daphnia magna* exposed to glyphosate acid was 50 mg/L based on nominal concentration.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Lot/Batch #:	P30

Purity: 97.6%

2. Vehicle and/or positive control: Elendt M4

3. Test organism:

Species: *Daphnia magna*

Age: Neonates (< 24 h old)

Loading: 1 organism per vessel (glass beakers containing 80 mL test solution)

Source: [redacted]

4. Environmental conditions:

Temperature: 19.4 to 20.2°C

pH: 3.67-8.02 (new solutions)
3.46-8.00 (old solutions)

Dissolved oxygen: 9.2-9.2 mg O_2/L (dilution water, new)
8.8-9.2 mg O_2/L (test solutions, old)

Conductivity: 572-617 mg/L µS/cm (test solutions)

Hardness: 202.7-218.3 mg $CaCO_3$

Photoperiod: 16 hours light/8 hours dark, 20 minute dawn and dusk transition period; 480 lux

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The lethal and sub lethal effects of glyphosate acid on *Daphnia magna* were evaluated in a 21-day toxicity test performed under semi-static conditions. Ten replicates of one *Daphnia* per concentration were exposed to 12.5, 25, 50, 100, and 200 mg a.s./L nominal concentrations. In addition, 10 x 1 *Daphnia* were exposed to test medium without test substance (blank control). The *Daphnia* were randomly placed into the test beaker and exposed to the test item for 21 days. The test *Daphnia* were fed daily with cultured algae (*Chlorella vulgaris*).

A primary stock solution of 200 mg a.s./L was prepared on day 0 by dissolving 400 mg test item in 2000 mL of dilution water. On days 2, 4, 7, 9, 11, 14, 16, and 18 a primary stock solution of 100 mg a.s./L was prepared by dissolving 200 mg test item in 2000 mL dilution water. The test solutions were prepared by the addition of appropriate aliquots of the stock solutions to dilution water. At each renewal of the test solutions, the surviving P_0 generation of *Daphnia* were transferred to the new solutions. The F_1 generation of *Daphnia* were removed from each vessel and counted. The numbers of alive and dead F_1*Daphnia* were recorded.

2. Observations: Mortality of P_0 generation of *Daphnia* and observation for the presence of alive and dead offspring (termed F_1 generation) were recorded daily in each test vessel. At the end of the test, the length of each surviving P_0*Daphnia* was measured.

The pH was measured in each newly prepared test solution. The pH and dissolved oxygen concentration of two of the replicates of the old test solutions were measured after transfer of the P_0 generation of daphnids. Temperature measurements were recorded daily by means of a thermometer and hourly automatically. The concentration of glyphosate acid in the test solutions was determined on days 0, 2, 7, 9, 14, and 16. Old solutions were analysed on days 2, 7, 9, 14, and 21.

3. Statistical calculations: The reproduction and length data for each individual P_0 generation daphnid were entered into electronic data files and analysed using statistical procedures contained in the Brixham Environmental Laboratory computer programs 'STATS' (version 4.10) and 'EPA' (version1.04).

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II. RESULTS AND DISCUSSION

A: FINDINGS

The mean measured concentrations of glyphosate acid in the new test solutions ranged from 100 to 104% of the nominal values. The mean measured concentrations in the old test solutions ranged from 96 to 104% of the nominal values. On the basis of the analytical data the nominal concentrations were used for the calculation and reporting of all results.

The 21-day EC_{50} and NOEC values (based on nominal concentrations) are given in the table below:

Mortality	
21-day EC_{50}	100 (95 % confidence interval 77-142)
21-day NOEC	50
21-day LOEC	100
Reproduction	
21-day NOEC	100
21-day LOEC	200
Length	
21-day NOEC	100
21-day LOEC	200
Overall result	
21-day NOEC	**50**
21-day LOEC	100

B. OBSERVATIONS

The effects of glyphosate acid on *Daphnia magna* mortality and reproduction are shown in Table 8.3.2.1-1.

Table 8.3.2.1-1: Chronic toxicity of glyphosate acid to *Daphnia magna*

Nominal concentration (mg a.s./L)	Mean adult mortality [%]	Total number of offspring per parent	Total offspring	Mean adult length [mm]
Control	10	108± 20	1028	4.28
12.5	0	100±21	1003	4.40
25	0	84±12*	840	4.31
50	0	91±18	912	4.31
100	50	109±23	763	3.81
200	100	-	-	A

Mortality before day 21

* Statistically significant difference

In the dilution water control and test concentrations up to and including 100 mg a.s/L all surviving P_0 *Daphnia* generation had released their first offspring by day 10. There was no reproduction at the concentration of 200 mg a.s./L due to mortality of the P_0*Daphnia*.

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Also, all validity criteria according to OECD 211 were fulfilled, as immobility of adult daphnids was ≤20% in control groups and number of offspring was >60 for the duration of the exposure.

III. CONCLUSION

The overall 21-day NOEC for the reproduction of *Daphnia magna* exposed to glyphosate acid was 50 mg/L based on nominal concentration.

Annex point	Author(s)	Year	Study title
IIA 8.3.2.1./02		2011	AMPA (Aminomethylphosphonic acid): A semi-static life cycle toxicity test with the Cladoceran (*Daphnia magna*) Report No: -2010-327 Date: 2011-06-17 GLP: yes Not published

Guideline: OECD Guideline 211 (1998)
ASTM E 1193-97

Deviations to OECD 211: Survival in the negative control group was slightly below the 80% criteria in OECD 211. This small difference was not considered to have impacted the validity of the study because the surviving daphnids in the control replicates appeared normal and healthy through test end indicating that the mortality observed was attributed to incidental death and not the health of the organisms.

Dates of experimental work: 2011-02-07 to 2011-06-17

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Executive Summary

The effects of AMPA (aminomethylphosphonic acid) on the survival, growth and reproduction of *Daphnia magna* were evaluated in a 21-day reproduction test under semi-static conditions with renewal of test medium every 2 to 3 days. The reproduction test was performed using a geometric series five nominal test concentrations (7.5, 15, 30, 60 and 120 mg AMPA/L) and a dilution water control (negative control). 10 replicates with one daphnid each were prepared per test concentration and 20 replicates with one daphnid each for the control.

Parental *Daphnia magna* were observed on a daily basis for mortality, onset of reproduction and signs of toxicity. Body length and dry weights of surviving parental specimens were measured at the end of the exposure period. The number of juvenile daphnia produced in each vessel was counted three times per week and at test termination. Mean measured test concentrations were determined from samples of test media collected from each treatment and control group at test initiation, at the end of the first renewal cycle, at the beginning and end of the longest renewal cycle during the second week of the test, and at the beginning and end of the last renewal cycle (test termination).

AMPA was not detected in the control group. The mean measured concentrations of AMPA in samples collected during the test for each treatment group were 7.4, 15, 30, 57 and 120 mg AMPA/L, equivalent to 99, 100, 100, 95 and 100% of the nominal concentrations, respectively. Therefore, the results evaluation is based on nominal test concentrations. There was no significant mortality observed during the test when compared to the control. Treatment related effects on growth were observed at 60 and 120 mg AMPA/L.

There was significant decrease in mean neonate production observed in the 30, 60 and 120 mg AMPA/L treatment groups.

Survival in the negative control group was slightly below the 80% validity criterion required in the OECD 211 test guideline. However, this minor deviation is not considered to have had a significant impact on the validity of this study as the surviving daphnids in the control replicates appeared normal and healthy throughout the test suggesting that the mortality observed was most likely attributable to incidental death and not related to the health of the organisms.

Adult daphnids in the control group produced an average of 227 live young per surviving adult (CV = 11.6%), which is well above the validity criterion of ≥60 live young per surviving adult. Therefore, the study is considered valid according to OECD 211.

The overall no observed effect concentration (NOEC) based on reproduction (juvenile production) was determined to be 15 mg AMPA/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: AMPA (Aminomethylphosphonic acid)
Description: Solid
Lot/Batch #: GLP-0908-19998A-A
Purity: 98.7%

2. Vehicle and/or positive control: ASTM medium

3. Test organism:

Species: *Daphnia magna* Straus
Age: Neonates (< 24 h old)
Loading: 1 daphnid per 200 mL test medium
Source: [redacted]
Diet/Food: Daily; mixture of yeast, cereal grass media and trout chow ([redacted]) and suspension of *Pseudokirchneriella subcapitata*

4. Environmental conditions:

Temperature: 19.0 – 20.8 °C
Photoperiod: 16 hours light
Light intensity = 314 lux
pH: 7.1 – 8.6
Dissolved oxygen: 6.8 – 9.1 mg O_2/L
Conductivity: 274 – 391 µS/cm
Hardness: 132 - 140 mg $CaCO_3$/L

B: STUDY DESIGN AND METHODS

1. Experimental treatments: A 21-day reproductive toxicity test was conducted under semi-static conditions, with renewal of test medium every 2 to 3 days. *Daphnia magna* neonates (<24 hours old) were

exposed to nominal concentrations of 7.5, 15, 30, 60 and 120 mg AMPA/L in moderately hard dilution water (ASTM medium). In addition, a negative control group was prepared in parallel. Ten glass vessels (250 mL vessels containing 200 mL test medium each) were used per treatment group for the test item and 20 vessels for the control group. One daphnid (neonate < 24 hours old) was exposed per replicate (vessel).

2. Observations: The number of living, immobilised and dead parental *Daphnia magna* and the time to gravidity (presence of eggs in brood pouch) were observed on a daily basis. Body length and dry weights of surviving parental specimens were measured at the end of the exposure period (21 days).

The number of neonate daphnids was counted three days a week and their condition was recorded. The presence of unhatched eggs was recorded, when observed. Incidental mortality was also recorded, when occurred. At the end of the test, body length and dry weight of each surviving parental daphnid was measured.

The temperature, pH-values and the oxygen saturation were measured at test initiation, before and after the renewal of the test media in two replicate test chambers and at test termination. Hardness, alkalinity and specific conductance were measured in batch solutions of the negative (dilution water only) control and at the highest test item concentration at test initiation and on one renewal day each week and from pooled replicate solutions at test termination.

Analytical measurements were performed by using an HPLC method of analysis using samples taken from all test concentrations for the freshly prepared solutions, at the end of the first renewal cycle (old solution), and at the beginning and end of last renewal cycle. For the aged test media, samples were taken from 2 alternate replicates of each treatment and control group and pooled by treatment group.

3. Statistical calculations: Data were statistically tested using Chi-square and Fisher's Exact test (discrete-variable data; $\alpha = 0.05$) and Dunnett's t-test (one-tailed, normal distributed data; $\alpha = 0.05$). The NOEC was determined by visual interpretation of the results.

II. RESULTS AND DISCUSSION

A. FINDINGS

Concentrations of AMPA in the freshly prepared test solutions, sampled on Days 0, 9 and 19 ranged from 92.5 to 106% of the nominal concentrations. Concentrations of AMPA in the old test solutions sampled immediately prior to renewal on Days 2, 12 and at test termination on Day 21 ranged from 78.6 to 117% of the nominal concentrations.

The overall mean measured concentrations of AMPA during the test were 7.4, 15, 30, 57 and 120 mg AMPA/L, equivalent to 99, 100, 100, 95 and 100% of the nominal concentrations, respectively. Since the mean measured test concentrations were within the 80 – 120% of nominal test concentration, the results of the study are reported as nominal test concentrations.

The 21-day EC_{50} and NOEC values are given below based on nominal concentrations.

Endpoints	AMPA [mg/L]
EC_{50} (21 days) for parental survival and immobility	> 120
NOEC (21 days) for parental survival and immobility	120
EC_{50} (21 days) for reproduction (95% C.I.)	90 (84 – 94)
NOEC (21 days) for reproduction	15
EC_{50} (21 days) for growth (95% C.I.)	90 (84 – 94)
NOEC (21 days) for growth	30
Overall LOEC	30

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Overall NOEC	15

B. OBSERVATIONS

Survival in the 7.5, 15, 30, 60 and 120 mg AMPA/L treatment groups at test termination was 80, 100, 70, 100 and 90%, respectively. No significant differences were detected in any treatment group in comparison to the control (α = 0.05, Fisher's Exact test). In the 120 mg AMPA/L treatment group, all surviving parental daphnids appeared pale and smaller in comparison to the control organisms from Day 5 through test end.

The first day of brood production in the controls and in all AMPA treatments indicated no delay in the onset of egg production at any of the AMPA concentrations tested. No aborted or shed eggs were present in the control or in any of the AMPA treatments. No males or ephippia were observed during the test.

Adult daphnids in the 7.5, 15, 30, 60 and 120 mg AMPA/L treatment groups produced an average of 229, 213, 189, 169 and 59.6 live young per surviving adult, respectively. Dunnett's test indicated there was a statistically significant decrease in mean neonate production in the 30, 60 and 120 mg AMPA/L treatment groups (30, 57 and 120 mg AMPA/L as mean measured concentration) in comparison to the negative control (α = 0.05).

In the control group , the mean body length was 5.3 mm and mean dry weight was 0.99 mg. Daphnids in the 7.5, 15, 30, 60 and 120 mg AMPA/L treatment groups had mean lengths of 5.2, 5.2, 5.1, 5.3 and 4.3 mm, respectively, and mean dry weights of 0.99, 1.0, 0.97, 0.69 and 0.45 mg, respectively. Dunnett's test indicated a significant decrease in length in the 30 and 120 mg AMPA/L (30 and 120 mg AMPA/L as mean measured concentration) treatment groups in comparison to the negative control (α = 0.05).

However, the decreases noted in the 30 mg AMPA/L treatment group was not dose related. Dunnett's test indicated there was a statistically significant decrease in dry weight in the 60 and 120 mg AMPA/L (57 and 120 mg AMPA/L as mean measured concentration) treatment groups in comparison to the control (α = 0.05).

Table 8.3.2.1-2: Chronic toxicity of AMPA to *Daphnia magna*

	Control	AMPA [mg/L]				
		7.5	**15**	**30**	**60**	**120**
Mortality of adults after 21 d [%]	25	20	0	30	0	10
Mean number offspring per adult	227±26.3	229 ±24.8	213 ±26.6	189 ±19.7*	169 ±22.1*	59.6 ±13.4*
Mean length of offspring	5.3 ±0.14	5.2 ±0.16	5.2 ±0.12	5.1 ±0.16*	5.3 ±0.18	4.3 ±0.17
Mean dry weight of offspring	0.99 ±0.24	0.99 ±0.12	1.0 ±0.22	0.97 ±0.25	0.69 ±0.20*	0.45 ±0.15*

* Indicates a statistically significant decrease in comparison to the negative control (Dunnett's one-tailed test, α = 0.05).

After 21 days of exposure, survival in control group was 75%. Although survival in the negative control group was slightly below the 80% criterion in OECD 211, this small difference is not considered to have impacted the validity of this study. The surviving daphnids in the control replicates appeared normal and healthy through until test end indicating that the mortality observed was attributed to incidental death and not the health of the organisms. Adult daphnids in the control group produced an average of 227 live young per surviving adult (CV = 11.6%), well above the validity criterion of ≥60 live young per surviving adult. Therefore, the study is considered valid according to OECD 211.

III. CONCLUSION

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The overall no observed effect concentration (NOEC) based on reproduction was determined to be 15 mg AMPA/L. Chronic NOEC values >1 mg/L for aquatic invertebrates is categorized by Canton et al. (1991)[5] as practically nontoxic.

IIA 8.3.2.2 Chronic toxicity for representative species of aquatic insects

As glyphosate acid is not an insecticide or insect growth regulator, studies on the acute toxicity for aquatic insects are not required.

IIA 8.3.2.3 Chronic toxicity for representative species of aquatic gastropod molluscs

This is not a data requirement according to Regulation 1107/2009/EC.

IIA 8.3.3 Aquatic field testing

Due to the low aquatic toxicity, field testing to assess potential impacts to aquatic invertebrates is not required or triggered.

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[5] Canton et al. 1991. Catch-up operation on old pesticides. National Institute of Public Health and Environmental Protection, Bilthoven. Report no. 678801001.

IIA 8.4 Effects on algal growth and growth rate (2 species)

A summary of all available relevant and compliant data (including data already reviewed during the 2001 EU evaluation of glyphosate) for glyphosate, glyphosate salts, AMPA and HMPA is presented in Table 8.4-1.

Table 8.4-1: Toxicity of glyphosate acid, glyphosate IPA salt, K-salt, and the metabolites AMPA and HMPA to algae

Species	Test design	EC_{50} (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
		Glyphosate acid		
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} = 54 E_bC_{50} = 48	141896 1995/yes	96-00065
Pseudokirchneriella subcapitata	96 h static	ErC_{50} = 114.05	IIA 8.4/01 -D2.44/99 2000/yes	-
Pseudokirchneriella subcapitata	120 h static	E_rC_{50} (72 h) =19 E_bC_{50} (72 h) = 18 E_rC_{50} (120 h) =21 E_bC_{50} (120 h) = 17	IIA 8.4/02 5550/B 1995/yes	-
Pseudokirchneriella subcapitata	168 h static	E_bC_{50} (72 h) = 12.31 E_bC_{50} (168 h) = 12.65		95-00722 1987
Desmodesmus subspicatus	96 h static	E_bC_{50} (72 h) = 195.2 E_bC_{50} (96 h) = 70.3		95-00002 1990
Desmodesmus subspicatus	72 h static	E_rC_{50} (24 h) = 60 E_bC_{50} (72 h) = 46	-	95-00535 1995
Anabaena flos-aquae	120 h static	E_rC_{50} (72 h) = 22 E_bC_{50} (72 h) = 8.5 E_rC_{50} (120 h) = 38 E_bC_{50} (120 h) = 15	IIA 8.4/03 5698/B 1996/yes	-
Anabaena flos-aquae	168 h static	E_bC_{50} (72 h) = 17.5 E_bC_{50} (168 h) = 17.15		95-00738 1987
Nitzschia palea	96 h static	E_rC_{50} = 11.90 E_bC_{50} = 4.47	960606 1996/yes	97-00013
Skeletonema costatum	120 h static	E_rC_{50} (72 h) = 18 E_bC_{50} (72 h) =11 E_rC_{50} (120 h) = 24 E_bC_{50} (120 h) =12	IIA 8.4/04 5684/B 1996/yes	
Skeletonema costatum	168 h static	E_bC_{50} (72 h) = 1.25 E_bC_{50} (168 h) = 0.64	IIA 8.4/05 1092-02-1100-3 1987c/no	96-00455

Skeletonema costatum	96 h static	Glyph. BN-78-44 = 1.3 BN-78-45 140 Comp. #1, BN-78-46 15 % effluent Comp. #4, BN-78-49 19% effluent Comp. 5A. 4.4% effluent (cell number)	-73-4-031 1978/no	96-00235
Navicula pelliculosa	120 h static	E_rC_{50} (72 h) = 17 E_bC_{50} (72 h) =16 E_rC_{50} (120 h) = 17 E_bC_{50} (120 h) =17	IIA 8.4/06 5673/B 1996/yes	-
Navicula pelliculosa	168 h static	E_bC_{50} (72 h)= 43.42 E_bC_{50} (168 h) = 42.40	1092-02-1100-2 1987d/no	96-00454
Glyphosate-IPA salt				
Pseudokirchneriella subcapitata	96 h static	E_rC_{50} = 45.68 E_bC_{50} = 14.69	IIA 8.4/07 A-99-02-03 2002/yes	
Desmodesmus subspicatus	72 h static	E_rC_{50} = 241 E_bC_{50} = 41.1	80-91-2328-01-93 1993/yes	93-00002
Desmodesmus subspicatus	72 h static	E_rC_{50} = 128.8 E_bC_{50} = 56.7 E_yC_{50} = 65.3	IIA 8.4/08 -1874 2002/yes	-
Desmodesmus subspicatus	72 h static	E_rC_{50} = 166.0 **E_bC_{50} = 72.9**	-	95-00554 1994
Glyphosate K-salt				
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} = 114 E_bC_{50} =69 E_yC_{50} = 74	IIA 8.4/09 -2002-148 2003/yes	
AMPA				
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} = 200 E_bC_{50} = 110	IIA 8.4/10 232458 1998/yes	-
Desmodesmus subspicatus	72 h static	E_rC_{50} = 452 E_bC_{50} = 89.8	93006/01 1994/yes	94-00501
HMPA				
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} (72 h) >115 E_yC_{50} (72 h) >115	IIA 8.4/11 139A-396A 2011/yes	-

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph

Overview

Studies not submitted within the scope of the 2001 EU Evaluation of Glyphosate are summarised below. With regard to the studies submitted within the 2001 EU Evaluation of Glyphosate, it has to be pointed out that some of these studies do not meet current GLP standards and strongly deviate from current OECD 201 study design and validity criteria (1987a,b,c,d; 1978). Therefore, studies on the

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same algal species, which are compliant to current guidelines, are presented below. The endpoint of the previous 2001-EU Evaluation was based on a study conducted with the marine algal species *Skeletonema costatum,* (1987c). Apart from the fact that studies conducted on marine organisms do not address any current EU-requirement, the design of this non-GLP study strongly deviates from current EU and US guidelines. Therefore, for the purpose of completeness and to demonstrate that the toxicity of glyphosate and its metabolites to marine organisms is within the same range as for fresh water species, an existing, guideline compliant study on *S. costatum* is presented below (1996, IIA 8.4/04).

A comprehensive review of the inadequacies and lack of validity of 1987c and , 1978 *Skeletonema* studies is provided in Appendix II.

In line with that, the following endpoints are proposed for the risk assessment of glyphosate and its relevant metabolites on algae:

Table 8.4-2: Proposed endpoints for ecotoxicological risk assessment of algae exposed to glyphosate

Species	Test design	EC_{50} (mg a.s./L)	Reference
Glyphosate acid			
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} = 54 mg/L E_bC_{50} = 48 mg/L	141896 1995
Desmodesmus subspicatus	72 h static	E_bC_{50} (72 h) = 46 mg/L	95-00535 1995
Anabaena flos-aquae	120 h static	E_rC_{50} (120 h) = 38 mg/L E_bC_{50} (120 h) = 15 mg/L	IIA 8.4/03 5698/B 1996
Nitzschia palea	96 h static	E_rC_{50} = 11.90 mg/L E_bC_{50} = 4.47 mg/L	960606 1996
Skeletonema costatum	120 h static	E_rC_{50} (120 h) = 24 mg/L E_bC_{50} (120 h) = 12 mg/L	IIA 8.4/04 5684/B ., 1996
Navicula pelliculosa	120 h static	E_rC_{50} (120 h) = 17 mg/L E_bC_{50} (120 h) = 17 mg/L	IIA 8.4/06 5673/B 1996
Glyphosate-IPA salt			
Pseudokirchneriella subcapitata	96 h static	E_bC_{50} = 14.69 mg/L E_rC_{50} = 45.68 mg/L	IIA 8.4/07 A-99-02-03 2002
Desmodesmus subspicatus	72 h static	E_bC_{50} = 41.1 mg/L E_rC_{50} = 241 mg/L	80-91-2328-01-93 1993
Glyphosate K-salt			
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} = 114 mg/L E_bC_{50} = 69 mg/L E_yC_{50} = 74 mg/L	IIA 8.4/09 -2002-148 , 2003/yes

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AMPA			
Pseudokirchneriella subcapitata	72 h static	E_rC_{50}= 200 mg/L E_bC_{50} = 110 mg/L	IIA 8.4/10 232458 1998
Desmodesmus subspicatus	72 h static	E_rC_{50} = 452 mg/L E_bC_{50} = 89.8 mg/L	93006/01 1994
HMPA			
Pseudokirchneriella subcapitata	72 h static	E_rC_{50} (72 h) > 115 mg/L E_yC_{50} (72 h) > 115 mg/L	IIA 8.4/11 139A-396A 2011

Annex point	Author(s)	Year	Study title
IIA 8.4/01		2000	Acute toxicity of glifosate tecnico NUFARM to *Selenastrum capricornutum* Report No: D2.44/99 Date: 2000-01-03 GLP: yes Published

Guideline: OECD 201 (1993)

Deviations to OECD 201: The concentration of the test substance in the test media was measured only at the beginning of the study. However, glyphosate in aquatic studies has been shown to be stable and not measuring the glyphosate concentration at test termination does not affect the validity of this study.

Dates of experimental work: 1999-10-25 to 1999-11-12

Executive Summary

The toxicity of glyphosate acid to the green alga *Pseudokirchneriella subcapitata* was determined in a 96-hour, static toxicity test, performed at nominal glyphosate acid concentrations 5.6, 10, 32, 56, 100, 320, and 560 mg/L, corresponding to measured concentrations of 5.74, 9.81, 33.48, 58.55, 104.17, 325.42, and 585.52 mg glyphosate acid/L at the beginning of the test). A negative control group (culture medium only) was prepared in parallel. There were test vessels were 250 mL glass Erlenmeyer flasks containing 100 mL of test solution.

There were 3 replicate vessels prepared for the control and per treatment group. Each replicate vessel was inoculated with 1.6 x 10^4 algal cells/mL. Uninoculated vessels were also prepared for the control and each test group (blanks). The culture vessels were incubated at 24.3-24.4°C under continuous illumination for 96 h with algal cells kept in suspension by continuous shaking.

At daily intervals, samples of control or test media were taken from each test and blank vessel and the algal cell densities were determined by cell counting using a Neubauer improved haemocytometer. The pH-values of test media at the beginning and at the end of the test were measured and incubator temperature was recorded daily using a minimum-maximum thermometer. Glyphosate concentrations in the test media was measured only at the start of the test. The mean measured concentrations of glyphosate acid ranged from 80 to 120% of the nominal values. Glyphosate acid was not detected in the control group. The measured test concentration values were used for the calculation and reporting of all results. All validity criteria according to the OECD guideline 201 were fulfilled.

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The effective concentration of glyphosate acid causing 50% inhibition of growth (E_rC_{50}) in *Pseudokirchneriella subcapitata* after 96 hours when compared to the control was 114.05 mg test item/L, the no observed effect concentration (NOEC) was 104.17 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description	White powder
Lot/Batch #:	037-919-113
Purity:	954.9 g a.e./kg

2. Vehicle and/or positive control: Cell growth medium (OECD 201)

3. Test organism:

Species:	Green algae *Pseudokirchneriella subcapitata*
Source:	[redacted] USA
Initial cell concentration	1.6 x 10^4 cells/mL

4. Environmental conditions:

Temperature:	24.3-24.4°C
Photoperiod:	Continuous illumination, 7933 lux
pH:	7.17 – 7.22 at the start of the test 7.71 – 9.31 at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate to the green alga *Pseudokirchneriella subcapitata* was determined in a 96-hour static toxicity test performed at nominal glyphosate concentrations of 5.6, 10, 32, 56, 100, 320, and 560 mg glyphosate/L. A negative control group (algal media only) was also prepared. The test vessels (3 replicates per control and test concentrations) were 250 mL glass Erlenmeyer flasks containing 100 mL of test solution. Single blank vessels were also prepared for the control and at each test concentration which were not inoculated with algal cells.

A primary stock solution of nominal concentration of 10000 mg glyphosate/L was prepared by dissolving 1.0 g glyphosate acid in 100 mL distilled and deionised water. The primary stock solution was used to prepare secondary stocks solutions at 10, 100, and 1000 mg glyphosate acid/L. Appropriate aliquots of the secondary solutions were diluted to prepare the test concentrations, of which 100 mL volumes of each were dispensed to each test and blank vessel.

Each replicate vessel (except blank vessels) was inoculated with a algal cell density of 1.6 x 10^4 cells/mL, from an exponentially growing pre-culture . The culture vessels were incubated at 24.3 - 24.4°C under continuous illumination for 96 h. During incubation, the algal cells were kept in suspension by continuous shaking (129-130 rpm).

2. Observations: After 1, 2, 3, and 4 days, volumes of media were removed from all vessels and the algal cell densities were determined by cell counting using a Neubauer improved haemocytometer under and a

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light microscope (set to phase-contrast). The pH-values were determined in the control and each test medium at the beginning and at the end of the test. No morphological changes in algal cells were observed after 96 hours of exposure to glyphosate acid at any test concentration. The temperature in the incubator was measured daily with a minimum-maximum thermometer. The concentrations of glyphosate acid in the test solutions were measured at the start of the test only and results are based on initial measured concentrations.

3. Statistical calculations: The computer program used for descriptive statistics and estimating endpoints was STATGRAPHICS – Statistical Graphic System. Toxicity endpoints were calculated based on initial measured means, standard deviations and 95% confidence limits.

II. RESULTS AND DISCUSSION

A: FINDINGS

The E_rC_{50} (96 h), NOEC and LOEC values are given below based on mean measured concentrations.

Endpoint	Glyphosate acid [mg/L]
96 h NOEC	104.17
96h LOEC	325.42
96 h E_rC_{50}	114.05 (95% confidence limit 94.04-131.49)

B. OBSERVATIONS

The effective concentration of glyphosate acid causing 50% inhibition of growth rate after 96 hours when compared to the control was 114.05 mg glyphosate acid/L, the no observed effect concentration (NOEC) was 104.17 mg glyphosate acid/L. No morphological changes were observed after 96 hours of exposure to glyphosate acid at any test concentration.

Table 8.4-3: Mean cell densities and Percentage of inhibition of cell growth of *Pseudokirchneriella subcapitata* exposed for 72 and 96 hours to glyphosate acid

Test parameters	Control	Glyphosate acid [mg/L]						
	-	5.6	10	32	56	100	320	560
Mean cell densities (0-96 h) (x 10000 cells/mL)	740	732	723	723	707	473	48.4	23.4
Mean growth rate (0-96 h) [% of control]	-	99	98	98	96	64	7	3

The growth rate in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was ≤35%, for the whole test period it was ≤7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 96h E_rC_{50} for *Pseudokirchneriella subcapitata* exposed to Glyphosate acid was calculated to be 114.05 mg glyphosate acid/L, the no observed effect concentration (NOEC) based on growth rate was 104.17 mg glyphosate acid/L.

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Annex point	Author(s)	Year	Study title
IIA 8.4/02		1995	Glyphosate acid: Toxicity to the green alga *Selenastrum capricornutum* Report No: 5550/B Date: 1995-12-12 GLP: yes Published

Guideline: OECD 201 (1984), US EPA Guideline 540/09-82-020 (1982)

Deviations to OECD 201: To maintain an exponential growth rate over the extended test duration of 120 h, a nominal cell density of 3 x 10^3 cells/mL, which is slightly below the recommended density of 5 x 10^3 – 10^4 cells/mL for *P. subcapitata,* was selected.

Dates of experimental work: 1995-08-07 to 1995-08-12

Executive Summary

The toxicity of glyphosate acid to the green alga *Pseudokirchneriella subcapitata* was determined in a 120-hour static toxicity test performed at nominal glyphosate acid concentrations of 5.6, 10, 18, 32, 56, and 100 mg glyphosate acid/L. A negative control group (culture medium only) was also prepared.

The test was performed using six replicate control vessels and three replicate vessels for each concentration of glyphosate acid. Each replicate control and test vessel was inoculated with 0.370 mL of the inoculum culture to give a nominal cell density of 3 x 10^3 cells/mL. A blank vessel was used concurrently. The culture vessels were incubated at 24 ± 1°C under continuous illumination for 120 h.

The algal cell densities were determined by electronic particle counting after 1, 2, 3, 4, and 5 days. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start (preparation vessels) and at the end of the test (pooled media according to treatment). Glyphosate acid was not detected in the control group. The mean measured concentrations of glyphosate acid ranged from 100 to 111% of the nominal values. The ecotoxicologically endpoints were evaluated using the nominal test concentration. All validity criteria according to the OECD guideline 201 were fulfilled.

The 120-hour E_bC_{50} and E_rC_{50} for *Pseudokirchneriella subcapitata* exposed to glyphosate acid were calculated to be 17 and 21 mg glyphosate acid/L respectively. The 72-hour E_bC_{50} and E_rC_{50} were determined to be 18 and 19 mg glyphosate acid /L. The corresponding 72-hour NOE_bC and NOE_rC was 10 mg glyphosate acid/L (nominal).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid

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Description	White solid
Lot/Batch #:	P24
Purity:	95.6%

2. Vehicle and/or positive control: Cell growth medium acc. to [redacted] (1978)

3. Test organism:

Species:	Green algae *Pseudokirchneriella subcapitata* Korshikov
Source:	[redacted]
Initial cell concentration	3 x 10^3 cells/mL

4. Environmental conditions:

Temperature:	24.1-24.2°C (measured by thermometer) The hourly temperature measured automatically remained within 24±1°C
Photoperiod:	Continuous illumination
Light intensity	5030 lux
pH:	3.5 – 7.5 at the start of the test 3.6 – 8.9 at the end of the test

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to the green alga *Pseudokirchneriella subcapitata* was determined in a 120-hour, static toxicity test, performed at six nominal glyphosate acid concentrations of 5.6, 10, 18, 32, 56, and 100 mg glyphosate acid/L. A negative control group (culture medium without test item) was also prepared. The test vessels were conical glass flasks of 250 mL nominal capacity containing 100 mL of test solution.

A nominal 100 mg glyphosate acid/L stock solution was prepared by adding glyphosate acid directly to 2000 mL sterile culture medium. Appropriate aliquots of this stock solution were diluted to prepare the lower test concentrations of 5.6, 10, 18, 32, and 56 mg glyphosate acid/L. Then 100 mL volumes of control medium or the appropriate test medium were dispensed to each replicate test and control vessel. A blank vessel was also prepared for each treatment that would not be inoculated with algal cells. The test was performed in 6 replicate vessels for the control group and 3 replicate vessels for each concentration of glyphosate acid.

Each replicate test vessel was inoculated with 0.370 mL of the algal inoculum culture to give a nominal cell density of 0.300 x 10^4 cells/mL. The culture vessels were incubated at 24 ± 1°C under continuous illumination for 120 h. During incubation, the algal cells were kept in suspension by continuous shaking.

2. Observations: The algal cell densities were determined by electronic particle counting, using a Coulter counter. After 1, 2, 3, 4, and 5 days, samples were removed from all vessels including the blank vessels. The appropriate blank particle count was subtracted from that of the test culture to obtain the corrected cell density. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start (preparation flasks) and at the end (pooled replicates according to treatment)of the test.

3. Statistical calculations: One-way analysis of variance and Dunnett's post-hoc test. Median effective concentrations and its 95% confidence limits were determined by linear regression against log concentration.

II. RESULTS AND DISCUSSION

A: FINDINGS

Analytical data: The overall mean measured concentrations of glyphosate acid ranged from 100 to 111% of the nominal values. On the basis of the analytical results being within 80 and 120% of the nominal concentrations, the nominal test concentration values were used for the calculation and reporting of all results.

Endpoint	Glyphosate acid [mg/L]
72–h NOE_rC	10
72–h LOE_rC (p=0.05)	18
72-h E_rC_{50}	19 (95% confidence limit 14-25)
72–h NOE_bC	10
72–h LOE_bC (p=0.05)	18
72-h E_bC_{50}	18 (95% confidence limit 13-23)
120–h NOE_rC	10
120–h LOE_rC (p=0.05)	18
120-h E_rC_{50}	21 (95% confidence limit 16-28)
120–h NOE_bC	10
120–h LOE_bC (p=0.05)	18
120-h E_bC_{50}	17 (95% confidence limit 13-22)

B. OBSERVATIONS

Table 8.4-4: Mean cell densities and percentage of inhibition of cell growth of *Pseudokirchneriella subcapitata* exposed for 72, 96 and 120 hours to glyphosate acid

Test parameters	Control	Glyphosate acid [mg/L]					
	-	5.6	10	18	32	56	100
Mean cell densities (0-72 h) (x 10000 cells/mL)	73.4	79.1	74.5	2.05	0.143	0.021	0.033
Mean cell densities (0-96 h) (x 10000 cells/mL)	312	314	311	2.60	0.178	0.070	0.045
Mean cell densities (0-120 h) (x 10000 cells/mL)	567	605	568	4.20	0.478	0.138	0.172
Mean area under growth curve (0-72 h) [%]	-	108	104	8	-1	-1	-1
Mean area under growth curve (0-96 h) [%]	-	103	101	2	0	0	0
Mean area under growth curve (0-120 h) [%]	-	104	100	1	0	0	0
Mean growth rate (0-72 h) [%]	-	101	100	35	-13	-48	-40
Mean growth rate (0-96 h) [%]	-	100	100	31	-7	-21	-27
Mean growth rate (0-120 h) [%]	-	101	100	35	6	-10	-7

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The biomass in the control cultures increased by a factor of >16, the coefficient of variance (C of V) for section –by-section specific growth rates was ≤35%. For the whole test period the C of V was ≤7%. The validity criteria according to guideline OECD 201 were therefore fulfilled.

III. CONCLUSION

The 120-hour E_bC_{50} and E_rC_{50} toxicity values for *Pseudokirchneriella subcapitata* exposed to glyphosate acid were calculated to be 17 and 21 mg glyphosate acid/L respectively.

The 72-hour E_bC_{50} and E_rC_{50} were determined to be 18 and 19 mg glyphosate acid/L, respectively. The 72-hour NOE_bC and NOE_rC values were 10 mg/L (nominal), respectively.

Annex point	Author(s)	Year	Study title
IIA 8.4/03	[redacted]	1996	Glyphosate acid: Toxicity to blue-green alga *Anabaena flos-aquae* [redacted] Report No: [redacted]5698/B Date: 1996-11-08 GLP: yes Published

Guideline: OECD 201 (1984)
US EPA Guideline 540/09-82-020 (1982)

Deviations to OECD 201: None

Dates of experimental work: 1996-03-04 to 1996-03-09

Executive Summary

The toxicity of glyphosate acid to the blue-green alga *Anabaena flos-aquae* was determined in a 120-hour, static test. The test incorporated 8 nominal concentrations of glyphosate acid (0.75, 1.5, 3.0, 6.0, 12, 24, 48, 96 mg a.s./L) and a negative control group consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 100 mL of test solution.

The test was performed with 6 replicate vessel prepared for the control and 3 replicate vessels for each concentration of glyphosate acid. Each replicate test vessel was inoculated with 1.120 mL of the inoculum culture to give a nominal cell density of 2.05 x 10^4 cells/mL. Single blank vessels were also prepared for the control and each test concentration without algal cells. The culture vessels were incubated at 24±1°C under continuous illumination for 120 h.

The algal cell densities were determined by spectrophotometric absorbance analysis. With samples of control or test medium measured after 1, 2, 3, 4, and 5 days, the pH-values were determined in the test media at the beginning and at the end of the test. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

The mean measured concentrations of glyphosate acid ranged from 98 to 110% of the nominal values. Glyphosate acid was not detected in the control group. On the basis of the analytical results being within 80-120% of nominal, ecotoxicological endpoints were evaluated using nominal concentrations. All validity criteria according to the OECD guideline 201 were fulfilled.

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The 72 h E_bC_{50} for *Anabaena flos-aquae* exposed to glyphosate acid was 8.5 mg a.s./L (95% C.I. 2.6 to 28 mg glyphosate/L) and the 120 h $E_bC_{50}$was 15 mg glyphosate/L (95% C.I. 9.7 to 27.0 mg glyphosate/L). The 72 h $E_rC_{50}$was 22 mg/L (95% C.I. 8.8 to >96 mg glyphosate/L) and the 120 h E_rC_{50} was 38 mg/L (95% C.I. 20 to >96 mg glyphosate/L).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description	White solid
Lot/Batch #:	P24
Purity:	95.6%

2. Vehicle and/or positive control: Cell growth medium acc. to [redacted] (1978)

3. Test organism:

Species:	Blue-green alga *Anabaena flos-aquae*
Source:	[redacted] UK
Initial cell concentration	2.05 x 10^4 cells/mL

4. Environmental conditions:

Temperature:	24.1-24.2°C (measured by thermometer) The hourly temperature measured automatically remained within 24±1°C
Photoperiod:	Continuous illumination
Light intensity	3600 lux
pH:	3.5 – 7.2at the start of the test 3.6 – 8.2at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to the blue-green alga *Anabaena flos-aquae* was determined in a 120-hour, static toxicity test. The test incorporated 8 nominal concentrations of glyphosate acid (0.75, 1.5, 3.0, 6.0, 12, 24, 48, 96 mg a.s./L) and a negative control consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 100 mL of test solution.

A stock solution at a nominal concentration of 96 mg glyphosate/L was prepared by adding glyphosate acid directly to 2000 mL sterile culture medium. Appropriate aliquots of this stock solution were diluted to prepare the lower test concentrations of 0.75, 1.5, 3.0, 6.0, 12, 24, and 48 mg a.s./L. 100 mL of the appropriate test solution were dispensed to each test and blank vessel.

The test was performed in 6 replicate vessels of culture medium only for the control group and 3 replicate vessels for each concentration of glyphosate acid. Each replicate test vessel was inoculated with 1.120 mL of the inoculum culture to give a nominal cell density of 2.05 x 10^4 cells/mL. Single blank vessels were

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also prepared for the control and each test concentration without algal cells. The culture vessels were incubated at 24±1°C under continuous illumination for 120 h. During incubation, the algal cells were kept in suspension by continuous shaking.

2. Observations: The algal cell densities were determined by spectrophotometric absorbance, using a Uvikon 860 UV/visible spectrophotometer. After 1, 2, 3, 4, and 5 days, samples were removed from each control, test and blank vessel. The appropriate blank solution absorbance was subtracted from that of the test culture to obtain the algal absorbance reading. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

3. Statistical calculations: One-way analysis of variance, and Dunnett's procedure. Median effective concentrations and its 95% confidence limits were determined by linear regression against log concentration.

II. RESULTS AND DISCUSSION

A: FINDINGS

The mean measured concentrations of glyphosate acid ranged from 98 to 110% of the nominal values. On the basis of the analytical results being with 80 and 120% of the nominal test concentration, ecotoxicological endpoints were evaluated using the nominal concentrations.

Endpoint	Glyphosate acid [mg/L]
72–h NOE_rC	12
72–h LOE_rC	24
72-h E_rC_{50}	22 (95% confidence limit 8.8 to >96)
72–h NOE_bC	12
72–h LOE_bC	24
72-h E_bC_{50}	8.5 (95% confidence limit 2.6-28)
120–h NOE_rC	12
120–h LOE_rC	24
120-h E_rC_{50}	38 (95% confidence limit 20 to >96)
120–h NOE_bC	12
120–h LOE_bC	24
120-h E_bC_{50}	15 (95% confidence limit 9.7-27)

B. OBSERVATIONS

Glyphosate acid inhibited cell growth of the fresh water algae *Anabaena flos-aquae* after 120 h at test concentrations of 24, 48 and 96 mg a.s./L (nominal).

Results are presented in Tables below.

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Table 8.4-5: Mean areas under the growth curve of *Anabaena flos-aquae* exposed for 72 hours to glyphosate acid

Nominal concentration (mg a.s./L)	0-72 h		0-96 h		0-120 h	
	Mean area under growth curve	Deviation to control [%]	Mean area under growth curve	Deviation to control [%]	Mean area under growth curve	Deviation to control [%]
Control	0.4	-	1.5	-	3.5	
0.75	0.4	0.0	1.5	0.0	3.6	-2.9
1.5	0.3	25.0	1.5	0.0	3.6	-2.9
3.0	0.3	25.0	1.4	6.7	3.5	0.0
6.0	0.3	25.0	1.4	6.7	3.5	0.0
12	0.3	25.0	1.3	13.3	3.3	5.7
24	0.0*	100.0	0.0*	100.0	0.0*	100.0
48	0.0*	100.0	0.0*	100.0	0.0*	100.0
96	0.0*	100.0	0.0*	100.0	0.0*	100.0

* Significant difference from the culture control (α =0.05)

Table 8.4-6: Mean growth rates of *Anabaena flos-aquae* exposed for 72 hours to glyphosate acid

Nominal concentration (mg a.s./L)	0-72 h		0-96 h		0-120 h	
	Mean growth rate	Deviation to control [%]	Mean growth rate	Deviation to control [%]	Mean growth rate	Deviation to control [%]
Control	1.392	-	1.331	-	1.139	-
0.75	1.365	1.94	1.357	-1.95	1.145	-0.53
1.5	1.336	4.02	1.355	-1.80	1.139	0.00
3.0	1.328	4.60	1.344	-0.98	1.141	-0.18
6.0	1.321	5.10	1.342	-0.83	1.144	-0.44
12	1.299	6.68	1.321	0.75	1.138	0.09
24	1.231*	11.57	0.216*	83.77	0.251*	77.96
48	0.231*	83.41	0.173*	87.00	0.139*	87.80
96	0.231*	83.41	0.173*	87.00	0.139*	87.80

* Significant difference from the culture control (α = 0.05)

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was ≤35%, for the whole test period it was ≤7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h E_bC_{50} for *Anabaena flos-aquae* exposed to glyphosate acid was 8.5 mg a.s./L (95% C.I. 2.6 to 28 mg a.s./L) and the 120 h $E_bC_{50}$was 15 mg a.s./L (95% C.I. 9.7 to 27.0 mg a.s./L) (nominal). The 72 h $E_rC_{50}$was 22 mg/L (95% C.I. 8.8 to >96 mg a.s./L) and the 120 h E_rC_{50} was 38 mg/L (95% C.I. 20 to >96 mg a.s./L) (nominal).

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Annex point	Author(s)	Year	Study title
IIA 8.4/04	[redacted]	1996	Glyphosate acid: Toxicity to the marine alga *Skeletonema costatum* [redacted] Report No: [redacted] 5684/B Date: 1996-11-08 GLP: yes Published

Guideline: OECD 201 (1984)
US EPA Guideline 540/09-82-020 (1982)

Deviations: None

Dates of experimental work: 1996-02-05 to 1996-02-10

Executive Summary

The toxicity of glyphosate acid on the marine alga *Skeletonema costatum* was determined in a 120-hour, static test. The test incorporated 8 nominal concentrations of glyphosate acid (1.0, 1.8, 3.2, 5.6, 10, 18, 32, and 56 mg a.s./L) and a negative control consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 100 mL of test solution.

The test was performed with 6 replicate vessel prepared for the control and 3 replicate vessels for each concentration of glyphosate acid. Each replicate test vessel was inoculated with 1.250 mL of the inoculum culture to give a nominal cell density of 1.00 x 10^4 cells/mL. The culture vessels were incubated at 20±1°C with a photoperiod of 16 hours light for 120 h.

The algal cell densities were determined using a Coulter counter. After 1, 2, 3, 4, and 5 days, samples were removed from each test and blank vessel. The appropriate blank particle count was subtracted from that of the test culture to obtain the cell density. pH values were determined in the test media at the beginning and at the end of the test. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

Glyphosate acid was not detected in the control group. The mean measured concentrations of glyphosate acid ranged from 94 to 106% of the nominal values.

On the basis of the analytical results being within 80-120% of nominal, ecotoxicological endpoints were evaluated using nominal concentrations. All validity criteria according to the OECD guideline 201 were fulfilled.

The 72 h E_bC_{50} for *Skeletonema costatum* exposed to glyphosate acid was 11 mg/L (95% C.I. 7.1 to 20 mg a.s./L) and the 120 h E_bC_{50} was 12 mg/L (95% C.I. 7.6 to 19 mg a.s./L); the 72 h E_rC_{50} was 18 mg/L (95% C.I. 10 to 42 mg a.s./L) and the 120 h E_rC_{50} was 24 mg/L (95% C.I. 12 to >56 mg a.s./L) (nominal).

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Glyphosate acid
Description	White solid
Lot/Batch #:	P24
Purity:	95.6%
2. Vehicle and/or positive control:	Cell growth medium (1980)
3. Test organism:	
Species:	Marine alga *Skeletonema costatum*, strain CCAP 1077/1C
Source:	UK
Initial cell concentration	1.00 x 10^4 cells/mL
4. Environmental conditions:	
Temperature:	20.0-20.1°C (measured by thermometer) The hourly temperature measured automatically remained within 20±1°C
Photoperiod:	16 h light
Light intensity:	4340 lux
pH:	7.1 – 8.1 at the start of the test 8.1 – 8.8 at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to the marine alga *Skeletonema costatum* was determined in a 120-hour, static test. The test incorporated 8 nominal concentrations of glyphosate acid (1.0, 1.8, 3.2, 5.6, 10, 18, 32, and 56 mg a.s./L) and a control consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 100 mL of test solution.

A stock solution of nominal concentration of 56 mg a.s./L was prepared by adding glyphosate acid directly to 2000 mL sterile culture medium. Appropriate aliquots of this stock solution were diluted to prepare the lower test concentrations of 1.0, 1.8, 3.2, 5.6, 10, 18, and 32 mg a.s./L. 100 mL of the appropriate test solution were dispensed to each test and blank vessel.

The test was performed in 6 replicate cultures of the culture medium control and 3 replicate cultures of each concentration of glyphosate acid. Each replicate test vessel was inoculated with 1.250 mL of the inoculum culture to give a nominal cell density of 1.00 x 10^4 cells/mL. The culture vessels were incubated at 20±1°C for 120 h. During incubation, the cells were kept in suspension by continuous shaking.

2. Observations: The cell densities were determined by electronic particle counting, using a Coulter counter. After 1, 2, 3, 4, and 5 days, samples were removed from each test and blank vessel. The appropriate blank particle count was subtracted from that of the test culture to obtain the cell density. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system.

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The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

3. Statistical calculations: One-way analysis of variance, and Dunnett's procedure. Median effective concentrations and its 95% confidence limits were determined by linear regression against log concentration.

II. RESULTS AND DISCUSSION

A: FINDINGS

The mean measured concentrations of glyphosate acid ranged from 94 to 106% of the nominal values. On the basis of the analytical results being with 80 and 120% of the nominal test concentration, ecotoxicological endpoints were evaluated using the nominal concentrations.

Endpoint	Glyphosate acid [mg/L]
72–h NOE_rC	1.8
72–h LOE_rC	3.2
72-h E_rC_{50}	18 (95% confidence limit 10 to 42)
72–h NOE_bC	1.8
72–h LOE_bC	3.2
72-h E_bC_{50}	11 (95% confidence limit 7.1-20)
120–h NOE_rC	10
120–h LOE_rC	18
120-h E_rC_{50}	24 (95% confidence limit 12 to >56)
120–h NOE_bC	1.8
120–h LOE_bC	3.2
120-h E_bC_{50}	12 (95% confidence limit 7.6-19)

B. OBSERVATIONS

Glyphosate acid inhibited cell growth of the marine algae *Skeletonema costatum* after 120 h at test concentrations of 18, 32 and 56 mg glyphosate acid/L; mean area under growth curve was affected at 10, 18, 32 and 56 mg glyphosate acid/L.

Results are presented in the Table below.

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Table 8.4-7: Mean cell densities and percentage of inhibition of cell growth of *Skeletonema costatum* exposed for 120 hours to glyphosate

	Control	Glyphosate acid [mg/L]							
Test parameters	**-**	**1.0**	**1.8**	**3.2**	**5.6**	**10**	**18**	**32**	**56**
Mean areas under growth curve (0-72 h)	37.4	38.0	38.9	29.5*	34.2	17.9*	2.8*	2.3*	1.5*
Mean areas under growth curve (0-120 h)	162.2	162.7	163.3	149.5*	156.9	132.1*	7.1*	4.0*	2.2*
Mean areas under growth curve (0-72 h) [%]	-	102	104	79	92	48	8	6	4
Mean areas under growth curve (0-120 h) [%]	-	100	101	92	97	81	4	2	1
Mean growth rates (0-72 h)	1.423	1.433	1.443	1.322*	1.387	1.111*	0.362*	0.295*	0.188*
Mean growth rates (0-120 h)	0.882	0.879	0.869	0.873	0.875	0.905	0.315*	0.115*	0.055*
Mean growth rates (0-72 h) [%]	-	101	101	93	97	78	25	21	13
Mean growth rates (0-120 h) [%]	-	100	99	99	99	103	36	13	6

* Significant difference from the culture control (α =0.05)

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was ≤35%, for the whole test period it was ≤7%. The validity criteria according to guideline OECD 201 were therefore fulfilled.

III. CONCLUSION

The 72 h E_bC_{50} for *Skeletonema costatum* exposed to glyphosate acid was 11 mg/L (95% C.I. 7.1 to 20 mg a.s./L) and the 120 h E_bC_{50} was 12 mg/L (95% C.I. 7.6 to 19 mg a.s./L); the 72 h E_rC_{50} was 18 mg/L (95% C.I. 10 to 42 mg a.s./L) and the 120 h E_rC_{50} was 24 mg/L (95% C.I. 12 to >56 mg a.s./L) (nominal).

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Annex point	Author(s)	Year	Study title
IIA 8.4/05	[redacted]	1987	The Toxicity of Glyphosate Technical to *Skeletonema costatum* [redacted] USA Report No: 1092-02-1100-3 Date: 1987-04-27 GLP: no not published

Guideline: Guideline 123-2, U.S. EPA – FIFRA (Growth and Reproduction of Aquatic Plants, Tier 2)

Deviations to OECD 201: Algal biomass was not measured after day 1. A 7 day old inoculum culture was used instead of a 2-4 day old culture. Samples for analysis of test concentration at test termination were not obtained from blanks but by removal of algae by filtration. pH values were not reported. The levels of acceptable control variance and growth rate were not achieved in the study. A full review of the impact of the deviations concluding the lack of validity is provided in Appendix II.

Dates of experimental work: 1987-04-20 to 1987-04-27

Executive Summary

The toxicity of glyphosate acid on the marine alga *Skeletonema costatum* was determined in a 168-hour static test. The test incorporated 6 nominal concentrations of glyphosate acid (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.s./L) and a negative control consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 50 mL of test solution.

The test was performed with 3 replicate vessels for each concentration of glyphosate acid and the control. Each replicate test vessel was inoculated with 0.367 mL of a 7 day old pre-culture of *Skeletonema costatum* to give a nominal cell density of 1.00×10^4 cells/mL. The salinity was adjusted to 30‰ at test initiation and the culture vessels were incubated at 20±2°C with a photoperiod of 14 hours light for 168 h.

The algal cell densities were determined using a Coulter counter. After 2, 3, 4, and 7 days, samples were removed from each test vessel. The organic calibration medium was subtracted from that of the test culture to obtain the cell density. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

Glyphosate acid was not detected in the control group. The mean measured concentrations of glyphosate acid ranged from 107 to 240% of the nominal values (measured: 0.24, 0.28, 0.48, 1.79 and 3.42 mg a.s./L, respectively).

On the basis of the analytical results, ecotoxicological endpoints were evaluated using mean measured concentrations. All validity criteria according to the OECD guideline 201 were fulfilled.

The 72 h E_bC_{50} for *Skeletonema costatum* exposed to glyphosate acid was 1.25 mg a.s./L and the 168 h E_bC_{50} was 0.64 mg a.s./L (mean measured). However, this study did not fulfil the validity criteria according to guideline OECD 201 and this study is not considered valid for risk assessment purposes (see Appendix II).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Glyphosate acid
Description:	White solid
Lot/Batch #:	NBP-3594465
Purity:	96.6%
Water solubility	1.2% at 25°C

2. Vehicle and/or positive control: Synthetic sea water containing 30 g/L commercial salt mix ([redacted])

3. Test organism:

Species:	Marine alga *Skeletonema costatum*
Initial cell concentration	1.00 x 10^4 cells/mL
Source:	[redacted]

4. Environmental conditions:

Temperature:	20 ± 2°C
Photoperiod:	14 h light
Light intensity	4306 ± 650 Lux
Salinity:	30‰
pH:	Not stated

B. STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to the marine alga *Skeletonema costatum* was determined in a 168-hour, static test. The test incorporated 6 nominal concentrations of glyphosate acid (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.s./L) and a control consisting of culture medium without test item. The test vessels were conical glass flasks of 250 mL nominal capacity containing 50 mL of test solution.

A stock solution of nominal concentration of 5.0 mg a.s./mL was prepared by adding glyphosate acid to 50 mL sterile deionized water. Stock solutions of 0.5 and 0.05 mg/mL were prepared by serial dilution. 50 mL of the appropriate test solution were dispensed to synthetic seawater (prepared by adding approximately 30 grams of a commercial salt mix to 1 L of distilled deionised water).

The test was performed in 3 replicate cultures of the culture medium control and of each concentration of glyphosate acid. Each replicate test vessel was inoculated with 0.367 mL of a 7 day old pre-culture of *Skeletonema costatum* to give a nominal cell density of 1.00 x 10^4 cells/mL. The salinity was adjusted to 30‰ at test initiation and the culture vessels were incubated at 20±2°C for 120 h. During incubation, the flasks were shaken manually each working day.

2. Observations: The cell densities were determined by electronic particle counting, using a Coulter counter. After 2, 3, 4, and 7 days, samples were removed from each test vessel. The organic calibration medium was subtracted from that of the test culture to obtain the cell density. The temperature was measured daily and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

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3. Statistical calculations: Median effective concentrations and its 95% confidence limits were determined by plotting the log of test concentration against percent inhibition expressed as Probit.

II. RESULTS AND DISCUSSION

A. FINDINGS

The mean measured concentrations were 0.24, 0.28, 0.48, 1.79 and 3.42 mg glyphosate/L, corresponding to 240.0%, 140.0%, 120.0%, and 117.5%, 111.9% and 106.9% of the nominal test concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg a.s./L, respectively. Therefore, ecotoxicological endpoints were evaluated using measured concentrations of the test item.

Endpoint	Glyphosate acid (mean measured) [mg/L]
72-h E_bC_{50}	1.25 (95% confidence limit 0.62 to 4.42)
168-h E_bC_{50}	0.64 (95% confidence limit 0.21 to 1.70)

B. OBSERVATIONS

Glyphosate acid inhibited cell growth of the marine algae *Skeletonema costatum* after 72 and 168 hours at test concentrations of 0.48, 0.94, 1.79 and 3.42 mg glyphosate acid/L.

Results are presented in the Table below.

Table 8.4-8: Percentage growth inhibition of Skeletonema costatum exposed to glyphosate acid

Nominal concentrations [mg a.s./L]	Control	0.1	0.2	0.4	0.8	1.6	3.2
Measured concentrations [mg a.s./L]	**-**	**0.24**	**0.28**	**0.48**	**0.94**	**1.79**	**3.42**
Mean number of algae cells after 72 hours [x 1000 Cells/mL]	36.3	30.0	36.7	24.7	20.3	15.3	12.7
Mean inhibition (72 hours) [%]	-	17.4	-0.9	32.1	44.0	57.8	65.1
Mean number of algae cells after 168 hours [x 1000 Cells/mL]	360.7	327.3	410.7	250.7	76.3	24.0	15.7
Mean inhibition (168 hours) [%]	-	9.2	-13.6	30.5	78.8	93.3	95.7

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was >35%, for the whole test period it was ≤7%. Because the coefficient of variation for the section specific growth rate was > 35%, the validity criteria according to guideline OECD 201 were not met and this study is not considered valid for risk assessment purposes. For a detailed evaluation of the current study, see Appendix II.

III. CONCLUSION

The 72 h E_bC_{50} for *Skeletonema costatum* exposed to glyphosate acid was 1.25 mg a.s./L and the 168 h E_bC_{50} was 0.64 mg a.s./L (mean measured). However, this study did not fulfil the validity

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criteria according to guideline OECD 201 and this study is not considered valid for risk assessment purposes.

Annex point	Author(s)	Year	Study title
IIA 8.4/06	[redacted]	1996	Glyphosate acid: Toxicity to freshwater diatom *Navicula pelliculosa* [redacted] Report No: [redacted]5673/B Date: 1996-11-10 GLP: yes Published

Guideline:	OECD 201 (1984) US EPA Guideline 540/09-82-020 (1982)
Deviations to OECD 201:	None
Dates of experimental work:	1996-01-29 to 1996-02-03

Executive Summary

The toxicity of glyphosate acid to the freshwater diatom *Navicula pelliculosa* was determined in a 120-hour, static toxicity test incorporating 8 nominal concentrations of glyphosate acid (1.8, 3.2, 5.6, 10, 18, 32, 56, and 100 mg a.s./L) and a negative control group (culture medium without test item) also prepared in parallel. The test vessels were 250 mL conical glass flasks containing 100 mL of control or test medium.

The test was performed in 6 replicate vessels for the control group and 3 replicate vessels for each concentration of glyphosate acid. Each replicate test vessel was inoculated with 0.915 mL of the inoculum culture to give a nominal cell density of 0.300 x 10^4 cells/mL. A single blank vessel was also prepared for the control and each test concentration without algal cells (blank vessel). The culture vessels were incubated at 24±1°C under continuous illumination for 120 h.

The cell densities were determined by electronic particle counting, using a Coulter counter. After 1, 2, 3, 4, and 5 days, samples were removed from each control, test and blank vessel. The appropriate blank particle count was subtracted from that of the test culture to obtain the cell density. The % inhibition in biomass and rate relative to the control group was then determined. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

The mean measured concentrations of glyphosate acid ranged from 106 to 111% of the nominal values. Glyphosate acid was not detected in the control group. On the basis of the analytical results being within 80 and 120% of the nominal test concentration, ecotoxicological endpoints were evaluated using the nominal concentrations.

The 72 h E_bC_{50} for *Navicula pelliculosa* exposed to glyphosate acid was 16 mg/L (95% C.I. 12 to 22 mg a.s./L) and the 120 h E_bC_{50} was 17 mg/L (95% C.I. 13 to 24 mg a.s./L); the 72 h E_rC_{50} was 17 mg/L (95% C.I. 13 to 24 mg a.s./L) and the 120 h E_rC_{50} was 17 mg/L (95% C.I. 12 to 24 mg a.s./L).

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description	White solid
Lot/Batch #:	P24
Purity:	95.6%
2. Vehicle and/or positive control:	Cell growth medium acc. to (1978)
3. Test organism:	
Species:	Freshwater diatom *Navicula pelliculosa* strain UTEX 667
Source:	
Initial cell concentration	0.300 x 10^4 cells/mL
4. Environmental conditions:	
Temperature:	24.0-24.1°C (measured by thermometer) The hourly temperature measured automatically remained within 24±1°C
Photoperiod:	Continuous illumination, 4560 lux
pH:	3.7 – 8.3 at the start of the test 3.7 – 8.7 at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity of glyphosate acid to the freshwater diatom *Navicula pelliculosa* was determined in a 120-hour, static toxicity test. The test incorporated 8 nominal concentrations of glyphosate acid (1.8, 3.2, 5.6, 10, 18, 32, 56, and 100 mg a.s./L) and a negative control consisting of culture medium without test item. The test vessels were 250 mL conical glass flasks containing 100 mL of control or test medium.

The stock solution of nominal concentration of 100 mg a.s./L was prepared by adding glyphosate acid directly to 2000 mL sterile culture medium. Appropriate aliquots of this stock solution were diluted to prepare the lower test concentrations of 1.8, 3.2, 5.6, 10, 18, 32, and 56 mg a.s./L. 100 mL of the appropriate test solution were dispensed to each control, test and blank vessel.

The test was performed in 6 replicate cultures of the culture medium control and 3 replicate cultures of each concentration of glyphosate acid. Each replicate test vessel was inoculated with 0.915 mL of the inoculum culture to give a nominal cell density of 0.300 x 10^4 cells/mL. A single blank vessel was also prepared for the control and each test concentration without algal cells (blank vessel). The culture vessels were incubated at 24±1°C under continuous illumination for 120 h. During incubation, the cells were kept in suspension by continuous shaking.

2. Observations: The cell densities were determined by electronic particle counting, using a Coulter counter. After 1, 2, 3, 4, and 5 days, samples were removed from each control, test and blank vessel. The appropriate blank particle count was subtracted from that of the test culture to obtain the cell density. The % inhibition in growth (biomass and rate) relative to the control group was then determined. The pH-

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values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and hourly with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

3. Statistical calculations: Probit analysis was used to calculate the EC_{50} values. One-way analysis of variance, and Dunnett's post-hoc test was used to determine significant differences between treatments and the control.

II. RESULTS AND DISCUSSION

A: FINDINGS

The mean measured concentrations of glyphosate acid ranged from 106 to 111% of the nominal values. On the basis of the analytical results being within 80 and 120% of the nominal test concentrations, the ecotoxicological endpoints were evaluated using the nominal test concentrations. The E_bC_{50} and E_rC_{50} (72 and 120 h) and corresponding NOEC and LOEC values are given below based on nominal concentrations.

Endpoint	**Glyphosate acid [mg/L]**
72–h NOE_rC	18
72–h LOE_rC	32
72-h E_rC_{50}	17 (95% confidence limit 13-24)
72–h NOE_bC	3.2
72–h LOE_bC	5.6
72-h E_bC_{50}	16 (95% confidence limit 12-22)
120–h NOE_rC [A]	18
120–h LOE_rC	32
120-h E_rC_{50}	17 (95% confidence limit 12 to 24)
120–h NOE_bC [B]	<1.8
120–h LOE_bC	1.8
120-h E_bC_{50}	17 (95% confidence limit 13-24)

[A] Effects observed in the 5.6 mg a.s./L test concentrations were due to growth enhancement. No inhibitory effects were observed below the nominal 32 mg a.s./L test concentration inhibitory effects were observed below the nominal 32 mg test concentration.

B. OBSERVATIONS

Glyphosate inhibited cell growth of the fresh water diatom *Navicula pelliculosa* after 120 h at test concentrations of 32, 56 and 100 mg glyphosate acid/L in terms of area under growth curve and growth rates.

Table 8.4-9: Mean cell densities, area under growth curve and mean growth rates of *Navicula pelliculosa* exposed for 120 hours to glyphosate acid

Test parameters	Control	Glyphosate acid [mg/L]							
	-	1.8	3.2	5.6	10	18	32	56	100
Mean algal cell density (72 h) [x 10^4 cells/mL]	18.2	22.0	27.6	32.0	29.8	10.9	0.071	0.005	0.006

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Test parameters	Control	Glyphosate acid [mg/L]							
	-	1.8	3.2	5.6	10	18	32	56	100
Mean algal cell density (120 h) [x 10^4 cells/mL]	170	197	156	166	160	187	0.237	0.212	0.147
Mean areas under growth curve (0-72 h)	11.0	12.1	16.7	22.6*	17.9*	5.8	-0.7*	-0.8*	-0.8*
Mean areas under growth curve (0-120 h)	197.7	285.8*	278.6*	311.3*	288.9*	178.4	-1.0*	-1.0*	-1.4*
Mean areas under growth curve (0-72 h) [%]	-	111	153	206	163	53	-6	-7	-7
Mean areas under growth curve (0-120 h) [%]	-	145	141	157	146	90	0	0	-1
Mean growth rates (0-72 h)	1.346	1.409	1.485	1.534	1.510	1.175	-0.504*	-1.366*	-1.309*
Mean growth rates (0-120 h)	1.255	1.284	1.237	1.250	1.243	1.274	-0.061*	-0.083*	-0.156*
Mean growth rates (0-72 h) [%]	-	105	110	114	112	87	-37	-102	-97
Mean growth rates (0-120 h) [%]	-	102	99	100	99	102	-5	-7	-12

* Significant difference from the culture control ($\alpha = 0.05$)

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was ≤35%, for the whole test period it was ≤7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h E_bC_{50} for *Navicula pelliculosa* exposed to glyphosate acid was 16 mg/L (95% C.I. 12 to 22 mg a.s./L) and the 120 h E_bC_{50} was 17 mg/L (95% C.I. 13 to 24 mg a.s./L); the 72 h E_rC_{50} was 17 mg/L (95% C.I. 13 to 24 mg a.s./L) and the 120 h E_rC_{50} was 17 mg/L (95% C.I. 12 to 24 mg a.s./L).

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Annex point	Author(s)	Year	Study title
IIA 8.4/07	███ ███	2002	A study on the Toxicity of Glyphosate isopropylamine salt 62.5% ███ Report No: A-99-02-04 Date: 2002-11-27 GLP: yes not published

Guideline: OECD Guideline 201, EEC Directive 92/69 C.3

Deviations to OECD 201: The pH-values of the algal medium recommended by, "Schlösser (1982). Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen (SAG) - List of Strains", were lower than reported in OECD 201. In correlation with the slightly lower pH values measured in concentration 100.0 mg/L there could be an effect on the growth rate of the algae.

Dates of experimental work: June 27, 2002 – July 19, 2002

Executive Summary

The effects of glyphosate isopropylamine salt on *Pseudokirchneriella subcapitata* were evaluated in a 96-hour static toxicity test at nominal concentrations of 4.27, 9.39, 20.66, 45.45 and 100 mg test item/L. A negative control group (culture medium only) was prepared in parallel. The test vessels were 300 mL Erlenmeyer glass flasks containing 100 mL of control or test medium .The initial cell concentration was 10^4 cells/mL. After 24, 48, 72 and 96 hours of growth, the numbers of viable cells for each test concentrations and control were determined and the growth inhibition (E_rC_{50}, E_bC_{50} and NOEC values) relative to the control was calculated.

The concentrations of glyphosate IPA salt in the test solutions were measured as concentrations of glyphosate acid at the start and at the end of the test in the (low, mid and high) 4.27, 20.66 and 100 mg test item/L treatments.

At the beginning of the test, a total recovery of 75.9% of the nominal concentration was measured in the test solution. At the end of the test (96 h), 64.2% recovery was measured in the test solution with algae, and 45.2% was found in the stability sample without algae. The average recovery in all water samples containing algae was 70.1% for Glyphosate isopropylamine salt 62.5%. Glyphosate acid was not detected in the control group.

After 72 hours, algae growth rates were significantly inhibited at 9.39 mg test item/L and higher. After 96 hours, there was significant inhibition observed at 20.66 mg test item/L and higher. For biomass, a significant effect was observed after 72 and 96 hours at 9.39 mg test item/L and higher. No growth inhibition was observed at or below 4.27 mg test item/L.

The 72 h and 96 h E_rC_{50} values for *Pseudokirchneriella subcapitata* exposed to glyphosate isopropylamine salt were calculated to be 31.70 and 32.01 mg test item/L, equivalent to 23.48 and 23.71 mg glyphosate acid/L (mean measured). The 72 h and 96 h E_bC_{50} for *P. subcapitata* exposed to glyphosate isopropylamine salt were calculated to be 9.25 and 10.20 mg test item/L, equivalent to 6.85 and 7.56 mg glyphosate acid/L (mean measured).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine salt
Description:	Light brown liquid
Lot/Batch #:	Tech L 020131
Purity:	62.66% Glyphosate isopropylamine salt
2. Vehicle and/or positive control:	SAG medium, Potassium dichromate
3. Test organism:	
Species:	*Pseudokirchneriella subcapitata*
Initial cell concentration	10^4 cells/mL
Source:	[redacted] Germany ([redacted])
4. Environmental conditions:	
Temperature:	21.7 – 25.0°C
Photoperiod:	24 h light
Light intensity	8082 lux
Light quality:	Universal white light
pH:	5.7 – 6.2

B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of a range finding test, the main test was performed with five concentrations, 4.27, 9.39, 20.66, 45.45 and 100 mg test item/L. A negative control (culture medium only) was prepared in parallel.

For each concentration and the control, four vessels were prepared using 300 ml Erlenmeyer flasks each containing 100 mL of control or test medium. The initial cell concentration was 10^4 cells/mL. The concentrations of glyphosate IPA salt in the test solutions were measured by HPLC as concentrations of glyphosate acid at the start and at the end of the test in the 4.27, 20.66 and 100 mg test item/L treatments. A stability sample was analysed from a test vessel without algae with the highest test item concentration at the end of the exposure period.

To maintain the algae in the suspension, all flasks were shaken continuously over the entire test period (100 ± 5 oscillations/min).

2. Observations: After 24, 48, 72 and 96 hours of growth, the algal cell densities in the control and test concentration vessels were determined using a Thoma counting chamber with a light microscope and the % growth inhibition (biomass and rate) relative to the control group was determined. This was achieved by plotting the mean value of the cell concentration (converted in log values) against the percentage growth inhibition to generate dose-response curves for each concentration. The concentrations resulting in 50% inhibition (E_rC_{50}, E_bC_{50}), were determined, as well as the NOEC. The pH-values were determined in the test media at the beginning and at the end of the test. The temperature in the incubator was measured daily with a thermometer, and continuously with an automatic recording system. The concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

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3. Statistical calculations: Probit analysis was used to calculate the EC_{50} values. One-way ANOVA, Cochran´s Test and subsequent Dunnett´s t-test was used to calculate whether there were significant differences between the growth of algae in the controls and the algae exposed to the various test item concentrations to establish NOE_rC and NOE_bC values.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: In the test, concentrations of glyphosate acid were determined. In stock solutions prepared at test start, measured concentrations were 81.9% of nominal concentrations .In test media at the beginning of the test, mean concentrations were 75.9% of nominal concentrations and at the end of the test (96 h), mean concentrations were 64.2%, of nominal with 45.2% found in the stability sample without algae (see table below). The average recovery in all water samples containing algae was 70.1% for Glyphosate isopropylamine salt. Therefore, results are based on mean measured concentrations.

Test item concentration (nominal) [mg/L]	glyphosate IPA (nominal) [mg/L]	glyphosate acid (mean measured) [mg/L		glyphosate IPA salt/L (mean measured) [mg/L]		% a.s. of nominal	
		-0.5 h	96 h	-0.5 h	96 h	-0.5 h	96 h
500 (Stock solution)	313.30	190.245		256.7		81.9	
Control	0	nd	nd	nd	nd	-	-
4.27	2.68	1.554	1.341	2.1	1.8	78.4	67.6
20.66	12.95	6.606	7.007	8.9	9.5	68.9	73.0
100	62.66	37.406	24.164	50.5	32.6	80.6	52.0
100 (stability sample without algae)	62.66	-	20.991	-	28.3	-	45.2

nd = not determined

The E_rC_{50}, E_bC_{50} and NOEC values are given below based on nominal and mean measured concentrations.

Endpoint	mg glyphosate IPA salt/L (nominal) [mg/L]	mg glyphosate IPA salt/L (mean measured) [mg/L]	mg glyphosate acid/L (mean measured) [mg/L]
0 - 72 h E_rC_{50}	45.23	31.70	23.48
0 - 96 h E_rC_{50}	45.68	32.01	23.71
0 - 72 h E_bC_{50}	13.20	9.25	6.85
0 - 96 h E_bC_{50}	14.69	10.30	7.63
NOEC	4.27	2.99	2.21

B. OBSERVATIONS

The results of the definitive test show that for algal growth rates, after 72 hours, these were significantly inhibited at nominal concentrations of 9.39 mg test item/L and higher. After 96 hours, significant inhibition was observed at 20.66 mg test item/L and higher.

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For biomass, after 72 and 96 hours, there were significant effects observed at nominal concentrations of 9.39 mg test item/L and higher.

In contrast no inhibition of the algae growth was found at or below a nominal concentration of 4.27 mg test item/L.

Table 8.4-10: Percentage inhibition of growth rate and biomass of to *Pseudokirchneriella subcapitata* exposed for 72 and 96 hours to glyphosate isopropylamine salt

Glyphosate isopropylamine salt formulation (nominal) [mg/L]	**Control**	**4.27**	**9.39**	**20.66**	**45.45**	**100.0**
Glyphosate isopropylamine salt (mean measured) [mg/L]		**1.95**	**4.12**	**9.19**	**19.96**	**41.56**
Glyphosate acid (mean measured) [mg/L]		**1.45**	**3.05**	**6.81**	**14.79**	**30.79**
Inhibition growth rate (0-72 h) [%]	-	1.6	6.6*	25.2*	64.8*	61.4*
Inhibition growth rate (0-96 h) [%]	-	-0.9	4.2	17.5*	53.4*	77.1*
Inhibition biomass (0-72 h) [%]	-	11.4	33.5*	70.7*	92.4*	91.9*
Inhibition biomass (0-96 h) [%]	-	3.2	27.1*	68.0*	94.9*	95.9*

* Significantly different from the control at $\alpha = 0.05$

For the reference item, the 96-hour E_bC_{50} was 0.497 mg/L and the 96-hour E_rC_{50} was 1.721 mg/L. These results were in agreement with what was expected on the basis of data shown in EEC Directive 92/69 method C.3.

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section-by-section specific growth rates was ≤35%, and for the whole test period was ≤7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h and 96 h E_rC_{50} values for *Pseudokirchneriella subcapitata* exposed to glyphosate isopropylamine salt were calculated to be 31.70 and 32.01 mg test item/L, equivalent to 23.48 and 23.71 mg glyphosate acid/L (mean measured). The 72 h and 96 h E_bC_{50} for *P. subcapitata* exposed to glyphosate isopropylamine salt was calculated to be 9.25 and 10.20 mg test item/L, equivalent to 6.85 and 7.56 mg glyphosate acid/L (mean measured).

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Annex point	Author(s)	Year	Study title
IIA 8.4/08	[redacted]	2002	Isopropylamine Salt of Glyphosate Acid: Toxicity to the Freshwater Alga *Scenedesmus subspicatus* [redacted] Report No: [redacted]-1874 Date: not stated GLP: yes Not published

Guideline: OECD Guideline 201 (2006)

Deviations to OECD 201: None

Dates of experimental work: Not stated

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Executive Summary

The toxicity of Isopropylamine (IPA) salt of glyphosate on the growth of the freshwater unicellular green alga *Desmodesmus subspicatus* (86.81SAG) was determined in a 72 hour static toxicity test, at nominal IPA salt of glyphosate concentrations of 2.88, 5.84, 11.7, 24.3, 48.6, 97.1, 194 and 388 mg test item/L, equivalent to 2.14, 4.33, 8.63, 18.0, 36.0, 72.0, 144 and 288 mg glyphosate acid/L. A negative control group (culture medium only) was prepared in parallel. The test vessels were 250mL conical flasks containing 100 mL of control or test medium. The vessels were continuously illuminated. Algal growth in each vessel was determined by measuring the cell density indirectly from absorbance at 540 nm in samples taken each day from all control and test vessels. Growth has been expressed as final cell numbers at 72 hours, as rate of increase in cell numbers per day and areas under the growth curves.

Samples of test media were analysed for glyphosate acid at the start and end of the test. Glyphosate acid was not detected in the control group. Mean measured concentrations of glyphosate acid were between 80-120% of nominal, therefore ecotoxicological endpoint evaluations were made based on nominal test item concentrations.

The 72 h E_rC_{50} value for *Desmodesmus subspicatus* exposed to glyphosate isopropylamine salt was calculated to be 128.8 mg test item/L. The 72 h E_bC_{50} for *D. subspicatus* exposed to glyphosate isopropylamine salt was calculated to be 56.7 mg test item/L and the 72 h E_yC_{50} was calculated to be 65.2 mg test item/L. The lowest NOEC of the IPA salt of glyphosate to *Desmodesmus subspicatus* measured over a 72-hour exposure period was 11.7 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate isopropylamine (IPA) salt
Lot/Batch #: 1002B
Purity: 97.1% as IPA salt

2. Vehicle and/or positive control: Cell growth medium (OECD 201)

3. Test organism:

Species: Algae *Desmodesmus subspicatus* CHODAT, strain:86.81SAG

Source: [redacted] Germany

Initial cell concentration 10^4 cells/mL

4. Environmental conditions:

Temperature: 19.5 – 22.5 C°

Photoperiod: Continuous illumination, 6670 - 7610 lux

pH: 5.45 – 10.25

B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of three range finding tests, the main test was performed with 8 nominal concentrations of IPA salt of glyphosate (2.88,5.84,11.7,24.3,48.6,97.1,194 and388 mg/L, equivalent to 2.14, 4.33, 8.63, 18.0, 36.0, 72.0, 144 and 288 mg glyphosate/L). A negative control (culture medium only) was prepared in parallel.

For each concentration, four vessels and for the control six vessels were prepared using 250 ml conical flasks each containing 100 mL of control or test medium. Additional vessels containing the test concentrations but with no algal inoculum were included in the second range finding test. The concentrations of test media were prepared as mg/L of the test item as received and were not corrected for purity (which was given as 97.1% as the IPA salt of glyphosate). The measured concentrations are corrected for the sample purity and expressed as mg glyphosate acid/L. The initial cell concentration was 10^4 cells/mL. The concentrations of glyphosate IPA salt in the test solutions were measured by HPLC as concentrations of glyphosate acid at the start and at the end of the test. Stability samples were analysed test vessels without algae.

To maintain the algae in the suspension, all flasks were shaken continuously over the entire test period (180 rpm).

2. Observations: The algal cell density was estimated by spectrophotometer. Samples were removed from each flask at daily intervals and the absorbance at 540 nm measured using a Perkin Elmer Lambda 5 Spectrophotometer.

After 24, 48 and 72 hours of growth, the algal cell densities in the control and test concentration vessels were determined by measuring cell density indirectly from absorbance at 540 nm in samples taken each day. Growth has been expressed as final cell number at 72 hours, rate of increase in cell numbers per day and areas under growth curves. The concentrations resulting in 50% inhibition (E_rC_{50}, $E_bC_{50,}$ E_yC_{50}), were determined, as well as the NOEC. The pH-values and temperature were determined in the test media at the beginning and at the end of the test and in one replicate flask from each treatment at 24 and 48 hours and at the end of the test. Following removal of the algae by centrifugation, the concentrations of glyphosate acid in the test solutions were measured at the start and at the end of the test.

3. Statistical calculations: E_rC_{50}, E_bC_{50}, and EC_{50}values for final cell numbers for the definitive test were calculated using the LC_{50} program of Stephan *et al.*, 1986. One-way ANOVA, Dunnett's test and the Bonferroni t-test was used to calculate whether there were significant differences between the growth of algae in the controls and the algae exposed to the various test item concentrations to establish NOE_rC and NOE_bC values.

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II. RESULTS AND DISCUSSION

A: FINDINGS

Analytical data: The mean measured content of the test item are shown in the table below; the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

glyphosate IPA (nominal) [mg/L]	glyphosate acid (nominal) [mg/L]	glyphosate acid (measured) [mg/L		glyphosate acid (mean measured) [mg/L]	glyphosate acid as % of nominal
		0 h	72 h		
Control	0	<0.26	<0.26	<0.26	-
2.88	2.14	2.14	1.85	2.00	93
5.84	4.33	4.08	2.80	3.44	79
11.7	8.64	8.22	7.93	8.07	94
24.3	18.0	12.5	17.6	15.1	84
49.6	36.0	31.5	34.7	33.1	92
97.1	72.0	58.9	66.5	62.7	87
194	144	135	137	136	94
388	288	280	280	280	97

Nd = not determined

The endpoint values given below are based on nominal concentrations of glyphosate-IPA salt.

Endpoint	Glyphosate-IPA [mg/L]
72 –h NOE_bC	11.7
72-h E_bC_{50}	56.7 (C.I.: 29.5 – 119.0)
72 –h NOE_yC	11.7
72-h E_yC_{50}	65.3 (C.I.: 38.5 – 120.3)
72 –h NOE_rC	24.3
72-h E_rC_{50}	128.8 (C.I.: 16.5 - > 100)

B. OBSERVATIONS

Glyphosate-IPA salt inhibited cell growth of the fresh water algae *Desmodesmus subspicatus* after 72 h within a test item concentration of 24.3 to 388 mg test item/L (nominal).

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Table 8.4-11: Calculation of the percentage of inhibition for the determination of the EC value (72 h)

Nominal concentration [mg test item/L]	Mean area under growth curve		Mean growth rate	
	(x10^6 cells/mL/h)	% inhibition	(cells x 10^6/mL/d)	% inhibition
Control	13.06	-	1.419	-
2.88	12.59	4	1.406	1
5.84	11.90	9	1.374	3
11.7	11.60	11	1.388	2
24.3	10.20	22*	1.319	7
48.6	8.44	35*	1.272	10*
97.1	7.86	40*	1.229	13*
194	1.27	90*	0.418	71*
388	0.48	96*	-0.012	101*

* significantly (α=0.05) different from the controls(1-tailed Bonferroni test)

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was >35%, and for the whole test period was ≤7%. As the coefficient of variation for the specific growth rate was >35%, the validity criteria according to guideline OECD 201 were not met and this study is not considered valid for risk assessment purposes.

III. CONCLUSION

The 72 h E_rC_{50} value for *Desmodesmus subspicatus* exposed to glyphosate isopropylamine salt was calculated to be 128.8 mg test item/L. The 72 h E_bC_{50} for *D. subspicatus* exposed to glyphosate isopropylamine salt was calculated to be 56.7 mg test item/L and the 72 h E_yC_{50} was calculated to be 65.2 mg test item/L. The lowest NOEC of the IPA salt of glyphosate to *Desmodesmus subspicatus* measured over a 72-hour exposure period was 11.7 mg/L.

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Annex point	Author(s)	Year	Study title
IIA 8.4/09	[redacted]	2003	MON 78623: A 72-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA (*Selenastrum capricornutum*) [redacted] Report No: [redacted]-2002-148 Date: 2003-02-11 GLP: yes not published

Guideline: OECD Guideline 201

EU Directive C3

Deviations to OECD 201: none

Dates of experimental work: 2002-10-18 to 2002-10-21

Executive Summary

The toxicity of the test item MON 78623 (glyphosate K-salt) on *Pseudokirchneriella subcapitata* was evaluated in a 72-hour static toxicity test at five nominal concentrations of 7.5, 15, 30, 60 and 120 mg test

item/L (mean measured: 7.1, 15, 30, 61, 122 mg test item/L). A negative control group (culture medium only) was prepared in parallel.

For each concentration, three replicates were prepared, with six replicates prepared for the control group. All vessels were inoculated with an initial algal cell density of 10^4 cells/mL An additional uninoculated replicate at the highest test concentration was included to verify test substance stability. The culture vessels were incubated at 22±1ºC on a mechanical shaker for 72 h.

After 24, 48, and 72 hours, mean cell counts were performed using an electronic particle counter. A primary counting standard containing *Pseudokirchneriella subcapitata* cells was prepared, and cell density was verified using a haemocytometer and a microscope. The inhibition of cell growth and reduction of cell growth rate were then calculated. The concentrations resulting in 50% reduction of growth rate (E_rC_{50}) and cell growth (E_bC_{50}) at 72 hours were determined with associated NOEC values. Measured concentrations of glyphosate K-salt in samples of the test media taken at test initiation were between 83 and 99% and in samples taken at test termination were 103 – 107%, confirming the test substance to be stable. Glyphosate K-salt was not detected in the control group.

At and above mean measured concentrations of 61 mg test item/L, cell density, biomass and growth were significantly inhibited by the test item compared to the control. All validity criteria according to the OECD guideline 201 were fulfilled.

The 72 h E_rC_{50} and the 72 h E_bC_{50} for *Pseudokirchneriella subcapitata* exposed to glyphosate K-salt were calculated to be 114 and 74 mg glyphosate K-salt/L, equivalent to 54.4 and 35.3 mg glyphosate acid/L, respectively. The NOEC was determined to be 30 mg glyphosate K-salt/L equivalent to 14.3 mg glyphosate acid/L. Based on cell density the 72 h EC_{50} was calculated to be 69 mg glyphosate K-salt /L, equivalent to 32.9 mg glyphosate acid/L. The NOEC was determined to be 30 mg glyphosate K-salt /L equivalent to 14.3 mg glyphosate acid/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 78623 (potassium salt of glyphosate)
Description:	Yellow liquid
Lot/Batch #:	GLP-0108-11688-F
Purity:	47.7% glyphosate
2. Vehicle and/or positive control:	OECD 201 medium
3. Test organism:	
Species:	*Pseudokirchneriella subcapitata*
Initial cell concentration	10^4 cells/mL
Source:	[redacted]
Acclimatisation period:	The pre-culture, which was used for the inoculation of the test cultures, was incubated 2 weeks under the conditions of the test.
4. Environmental conditions:	
Temperature:	22.0 – 22.3°C
Photoperiod:	24 h light
Light intensity	6500 – 8550 lux
pH:	6.9 – 8.1 (test initiation), 7.8 – 8.0 (test termination)

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the range finding test, the definitive algal growth inhibition test was performed with five concentrations of 7.5, 15, 30, 60 and 120 mg test item/L (achieving mean measured concentrations of 7.1, 15, 30, 61, 122 mg test item/L). In addition, algae were exposed to test medium without test substance (negative control). The algal medium recommended in OECD Guideline 201 was used. For each concentration, three parallel cultures were prepared in 250 mL Erlenmeyer flasks plugged with foam stoppers. To each test vessel, 100 mL of the algal suspension containing the test item preparation were added, with an initial cell density adjusted to 10^4 cells/mL. For the control group, six parallel test vessels were prepared with 100 mL volumes of culture media only. The culture vessels were incubated on a mechanical shaker in an environmental chamber for 72 h. During the incubation, the algal cells were kept in suspension by continuous shaking.

2. Observations: After 24, 48, and 72 hours, samples from all vessels were taken and algal cell counts were performed using an electronic particle counter. Use of the particle counter was validated / verified using a haemocytometer and a microscope.

Cell densities, areas under the growth curve, growth rates and percent inhibition were calculated using the SAS System for Windows v 8.02.

Temperature was recorded twice daily during the test, while the pH was measured in each test concentration and the control at test initiation and test termination. Light intensity (lux) was measured at test initiation.

3. Statistical calculations: EC_{50} values were calculated by non-linear regression Cell density, area under the growth curve and growth rate were evaluated for normality and homogeneity of variance (p = 0.05) using Shapiro-Wilks and Levene's test. Data were compared to the negative control using ANOVA and Dunnett's test ($\alpha = 0.05$). These data were used to determine the NOEC values.

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II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Chemical analyses were performed on samples of the test solutions to quantify glyphosate in the test solution. The recoveries at test initiation were between 83 and 99% and at test termination 103 – 107%, confirming the test substance to be stable. The ecotoxicological endpoints are based on the mean measured concentrations of 7.1, 15, 30, 61, 122 mg test item/L.

The E_rC_{50}, E_bC_{50}, EC_{50} toxicity values based on cell density and NOEC values are given below based on nominal concentrations.

Endpoint	Glyphosate K-salt [mg/L] (95% CL)	Glyphosate acid [mg/L]
EC_{50} (72 hours) based on cell density	69 (62 – 77)	32.9 (29.6 – 36.7)
E_rC_{50} (72 hours)	114 (111 – 118)	54.3 (52.9 – 56.3)
E_bC_{50} (72 hours)	74 (67 – 83)	35.3 (32.0 – 40.0)
NOEC (72 hours)	30	14.3

B. OBSERVATIONS

At and above mean measured concentrations of 61 mg test item/L, cell density, biomass and growth were significantly inhibited by the test item compared to the control see Table 8.2.6-15.

Table 8.4-12: Percentage reduction of growth rate and inhibition of cell growth of *Pseudokirchneriella subcapitata* exposed for 72 hours to glyphosate K-salt

Glyphosate K-salt [mg/L]	**Control**	**7.1**	**15**	**30**	**61**	**122**
Glyphosate acid [mg/L]	**-**	**3.4**	**7.2**	**14.3**	**29.1**	**58.2**
Cell density inhibition [%]	-	-14	-20	-6.8	33*	91*
Cell biomass inhibition [%]	-	-12	-17	-6.1	26*	88*
Cell growth inhibition [%]	-	-3.1	-4.3	-1.7	9.0*	54*

* Significant difference ($p < 0.05$) compared to the control according to Dunnett's test

The biomass in the control cultures increased by a factor of >16, the coefficient of variance for section specific growth rates was ≤35%, for the whole test period it was ≤7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h E_rC_{50} and the 72 h E_bC_{50} for *Pseudokirchneriella subcapitata* exposed to glyphosate K-salt were calculated to be 114 and 74 mg glyphosate K-salt/L, equivalent to 54.4 and 35.3 mg glyphosate acid/L, respectively. The NOEC was determined to be 30 mg glyphosate K-salt/L equivalent to 14.3 mg glyphosate acid/L. Based on cell density the 72 h EC_{50} was calculated to be 69 mg glyphosate K-salt/L, equivalent to 32.9 mg glyphosate acid/L. The NOEC was determined to be 30 mg glyphosate K-salt/L equivalent to 14.3 mg glyphosate acid/L.

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Annex point	**Author(s)**	**Year**	**Study title**
IIA 8.4./10		1998	Fresh Water Algal Growth Inhibition Test with (Aminomethyl)Phosphonic Acid Report No: 232458 Date: 1998-06-30 GLP: yes Not published

Guideline: OECD Guideline 201 (1984)
EEC Directive 92/69, Part C.3 (1992)
ISO International Standard 8692 (1989)

Deviations to OECD 201: None

Dates of experimental work: 1998-05-19 to 1998-05-29

Executive Summary

The effects of (Aminomethyl)phosphonic acid (AMPA) on *Pseudokirchneriella subcapitata* were evaluated in a 72-hour static toxicity test. After a range-finding test *Pseudokirchneriella subcapitata* were

exposed to nominal concentrations of 10, 22, 46, 100 and 220 mg test item/L. A negative control group (culture medium only) and a blank uninoculated vessel at the highest test rate were prepared in parallel.

For each test concentration and the control group, three (test concentrations) or six (control) replicates with 50 mL test solution and an initial cell density of 10^4 cells/mL were prepared in 100 mL vessels. The blank vessel was not inoculated. The culture vessels were incubated on a shaking plate for 72 h. After 24, 48, and 72 hours, algal cell densities in all vessels were determined based on spectrophotometrical measurements. Concentrations of AMPA in the 10, 46 and 220 mg AMPA/L treatments at test initiation were 99%, 100% and 102% of the nominal concentrations, respectively. At test termination, measured concentrations were 98%, 98% and 96% of the nominal concentrations, respectively. AMPA was not detected in the control group.

The concentrations resulting in 50% reduction of growth (rate (E_rC_{50}) and biomass (E_bC_{50}) were determined, as well as the associated NOEC values.

Statistically significant inhibition of cell growth was found at test concentrations of 100 mg/L and higher. The validity criteria according to guideline OECD 201 are fulfilled.

The 72 h E_rC_{50} and 72 h E_bC_{50}values for *Pseudokirchneriella subcapitata* exposed to Aminomethyl)phosphonic acid (AMPA) were calculated to be 200 mg AMPA/L and 110 mg AMPA/L, respectively. NOE_rC and NOE_bC were both determined to be 46 mg AMPA/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	(Aminomethyl)phosphonic acid (AMPA)
Description:	White powder
Lot/Batch #:	A010047101
Purity:	99%
2. Vehicle and/or positive control:	ISO-medium; Reference item: Potassium dichromate ($K_2Cr_2O_7$)
3. Test organism:	
Species:	*Pseudokirchneriella subcapitata,* strain: CCAP 278/4
Initial cell concentration:	10^4 cells/mL
Source:	[redacted]
Acclimatisation period:	4 days
4. Environmental conditions:	
Temperature:	22.5 – 23.0°C
Photoperiod:	24 h light
Light intensity	6000 - 7500 lux
Light quality	TLD-lamps of 18 Watt
pH:	8.5 (control), 6.0 – 6.8 (highest test concentration)
Hardness:	24 mg $CaCO_3$/L

B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of preliminary test results, the main test was performed with five concentrations: 10, 22, 46, 100 and 220 mg AMPA/L. A negative control group (culture medium only) was prepared in parallel. The test solutions were prepared using ISO-medium.

The culture vessels were incubated on a shaking plate for 72 hours. For each concentration, three parallel cultures were prepared in 100 ml all-glass vessels each containing 50 mL of control or test item preparation. All vessels were inoculated with 10^4 algal cells/mL. Additionally, for the highest test concentration one replicate without algae was provided (blank vessel). For the control group, six parallel test vessels were prepared.

2. Observations: After 24, 48, and 72 hours, mean algal cell densities for each test concentration and control were determined based on spectrophotometrical absorbance measurements from all test vessels following correction for the blank vessel and a linear calibration curve relating absorbance and cell density.

The concentrations resulting in 50% reduction in growth rates (E_rC_{50}) and 50% inhibition of growth in terms of biomass (E_bC_{50}) were determined, and the associated NOEC values.

The pH-values of test solutions were measured at test initiation and test termination. Temperature was recorded daily in a temperature-control vessel incubated alongside all other control and test vessels..

Concentrations of AMPA were determined using an HPLC method of analysis , in samples taken from three representative concentrations, 10, 46 and 220 mg test item/L at the start and end of the test.

3. Statistical calculations: The calculation of the E_rC_{50} and E_bC_{50} values were based on linear regression analysis of the percentages of growth inhibition of growth (biomass and rate) versus the logarithms of the corresponding nominal concentrations of the test substance. Data were compared to the negative control using ANOVA and Tukey's multiple comparisons test, Williams' test ($\alpha = 0.05$). These data were used to determine the NOEC values.

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II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Measured concentrations of AMPA at test initiation were 99%, 100% and 102% of the nominal 10, 46 and 220 mg AMPA/L concentrations, respectively. At test termination, concentrations were 98%, 98% and 96% of the nominal concentrations, respectively.

As the mean measured content of the test item ranged between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The E_rC_{50}, E_bC_{50} and NOEC values are given below based on nominal concentrations.

Endpoint	AMPA [mg/L]
0 - 72 h E_rC_{50} (95% CL)	200 (98 - 410)
0 - 72 h E_bC_{50} (95% CL)	110 (72 – 180)
NOE_rC	46
NOE_bC	46

CL confidence limit

Reference item: The 72-hour E_bC_{50} was 0.69 mg/L, the 72-hour E_rC_{50} was 1.7 mg/L (95% CI: 1.1 - 2.8 mg/L).

B. OBSERVATIONS

Mean cell densities: Cell densities were comparable to blank at nominal concentrations up to 46 mg/L while cell densities at 100 mg/L and 220 mg/L were increasingly reduced. At 220 mg/L almost no increase in cell densities were observed during the 72 hour test period.

Inhibition of cell growth (biomass): Inhibition of cell growth increased with increasing concentration of AMPA from a nominal concentration of 22 mg/L upwards. Statistically significant inhibition of cell growth was found at test concentrations of 100 mg/L and higher.

Inhibition of cell growth (rate) : Growth rates were in the range of the controls at the concentrations from 10to 46 mg/L during the 72-hour test period, whereas the growth rate of algae exposed to 100 and 220 mg/L were increasingly reduced. Statistically significant reduction of growth rate was found at test concentrations of 100 mg/L and higher.

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Table 8.4-13: Percentage reduction of growth rate and inhibition of cell growth of *Pseudokirchneriella subcapitata* exposed for 72 hours to AMPA

Test parameters	Control	AMPA [mg/L]				
	-	10	22	46	100	220
Mean cell densities (0-72 h) (x 10000 cells/mL)	67.8	73.0	67.6	64.5	41.5	5.4
Inhibition of biomass (0-72 h) [%]		-1.7	0.1	1.2	12.0	59.8
Inhibition of cell growth rate (0-72 h) [%]		-3.5	3.0	6.6	35.4	87.8

The biomass in the control cultures increased by a factor of > 16, the coefficient of variance for section by section specific growth rates was ≤ 35%, and for the whole test period was ≤ 7%. The validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h $E_rC_{50}$and E_bC_{50} toxicity values for *Pseudokirchneriella subcapitata* exposed to Aminomethylphosphonic acid (AMPA) were calculated to be 200 mg AMPA/L. and 110 mg AMPA/L, respectively. The NOE_rC and NOE_bC values were both determined to be 46 mg AMPA/L.

Annex point	Author(s)	Year	Study title
IIA 8.4/11		2011	HMPA (hydroxymethylphosphonic acid): A 72-Hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*) Report No: 139A-396A Date: 2011-10-11 GLP: yes not published

Guideline: OECD Guideline 201
EU Directive 92/69/EEC, Method C.3

Deviations from OECD 201: None

Dates of experimental work: 2011-04-04 to 2011-10-11

Executive Summary

The toxicity of HMPA (hydroxymethylphosphonic acid) on *Pseudokirchneriella subcapitata* were evaluated in a 72-hour static toxicity test at five nominal concentrations of 7.5, 15, 30, 60, and 120 mg HMPA/L, corresponding to mean measured concentrations of 7.3, 14, 29, 60 and 115 mg HMPA/L, respectively. In addition, a negative control group (culture medium only) was prepared in parallel.

Six replicate control vessels and three replicate test vessels per test concentration were prepared. Test vessels were 250 mL Erlenmeyer conical flasks each containing 100 mL of control or test media. All vessels were inoculated with an initial cell density of 10^4 cells/mL. The pH was adjusted to 7.4 - 7.5 with 10% HCl and 0.1N NaOH at test initiation. Test flasks were shaken continuously at 100 rpm. The temperature was recorded twice daily. The pH was measured in each treatment and control group at test termination.

After approximately 24, 48, and 72 hours, cell densities for each control and test concentration vessel were determined using an electronic particle counter ( ). Three counts per replicate were made. On the basis of the mean cell count, the percentage inhibition was determined and the ErC_{50} and E_yC_{50} values were calculated by non-linear regression using the algal growth curve, as well as the associated NOEC value. The concentrations of HMPA in the test solutions measured at the start and at the end of the test ranged between 93 and 100% of nominal concentrations. HMPA was not detected in the control group. Toxicity was based on nominal concentrations.

The validity criteria according to guideline OECD 201 are fulfilled.

The 72 h E_rC_{50} and E_YC_{50} toxicity values for *P. subcapitata* exposed to HMPA were both determined to be >115 mg HMPA/L. The NOEC was determined to be 60 mg HMPA /L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: HMPA (hydroxymethylphosphonic acid)
Description: solid
Lot/Batch #: GLP-1003-20448-A
Purity: 97.0%.

2. Vehicle and/or positive control: OECD medium

3. Test organism:

Species: *Pseudokirchneriella subcapitata*
Initial cell concentration 1×10^4 cells/mL
Source: [redacted]
Acclimatisation period: Not stated

4. Environmental conditions:

Temperature: 24 ± 2ºC
Light intensity Continuous illumination, 6030 - 7040 lux
pH: 7.0 – 7.2 at test start; 7.5 – 9.3 at test termination
Hardness: 1.044 mg (K_2HPO_4/L)

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of a range finding test, a main test was performed exposing actively growing *Pseudokirchneriella subcapitata* to HMPA at concentrations of 7.5, 15, 30, 60, and 120 mg HMPA/L. In addition, a negative control (culture medium only) was also prepared with algae exposed without test substance or other additives (negative control).

The control and test concentration vessels were incubated at 24 ±2ºC for 72 h on a mechanical shaker at 100 rpm. The replicate test vessels were 250 mL Erlenmeyer conical flasks, each containing 100 mL of control or test medium, with 6 control vessels and 3 test concentrations vessels prepared. Test algae were taken from a 3-day old stock culture and were aseptically added to the test medium to obtain a nominal initial concentration of 3000 cells/mL.

2. Observations: After 24, 48, and 72 hours, mean algal cell densities in samples of control or treated test media were determined using an electronic particle counter ([redacted]). Three counts per replicate were made. On the basis of the mean cell count, the percentage inhibition in algal cell growth relative to the control was determined. Prior to performing cell counts, the linearity of the instrument response was determined at settings previously established for *P. subcapitata*. The concentrations resulting in 50% reduction of growth rate (E_rC_{50}) and growth (biomass) inhibition (E_bC_{50}) were determined, as well as the associated NOAEC value.

The pH-value of the test item treatments and the controls was measured at test initiation and test termination. At test initiation, pH was adjusted to 7.4 - 7.5 with 10% HCl and 0.1N NaOH. The temperature was monitored continuously and recorded twice daily.

Samples of test media were taken at test initiation and test termination for analysis of the active ingredient content. At test initiation, samples were collected for each treatment and control group prior to distribution

of test solution into test chambers. At 72 hours, samples were collected from the pooled biological replicates from each respective treatment and control group. Samples were analysed using an HPLC method of analysis with mass detection (MS).

3. Statistical calculations: The calculation of the EC_{50} values was based on the percentages of growth (biomass) inhibition and the percentages of growth rate reduction versus (log) concentration using the linear regression method. The treatment groups were also compared to the negative control using Dunnett's test ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Measured concentrations of HMPA at test initiation were 106%, 94.1%, 99.4%, 104%, and 91.7% of the nominal concentrations (7.5, 15, 30, 60, and 120 mg HMPA/L) respectively. At test termination concentrations were, 88.0, 92.8, 92.4, 95.8, and 100% of the nominal concentrations, respectively. The overall mean measured test concentrations were 7.3, 14, 29, 60, and 115 mg HMPA/L, representing 97, 93, 97, 100, and 96% of the nominal concentrations, respectively. The results of the study were based on mean, measured test concentrations.

The pH tended to increase relative to increases in algal densities, which is typical for tests conducted with *Pseudokirchneriella subcapitata*.

The E_rC_{50}, E_bC_{50} and NOAEC values are given below based on mean measured concentrations.

Endpoint	mg HMPA/L (mean measured)
E_rC_{50} (72 hours) (95% CL)	>115 (n/a)[1]
E_yC_{50} (72 hours) (95% CL)	>115 (n/a)[1]
NOAEC	60

[1] 95% Confidence Interval not applicable.

B. OBSERVATIONS

HMPA inhibited cell growth of the fresh water algae *P. subcapitata* increasingly at increasing concentrations, resulting in a reduction in cell growth rate of 8% and an inhibition of cell growth (biomass) of 38% at 120 mg HMPA/L.

Table 8.4-14: Percentage reduction of growth rate and inhibition of cell growth (biomass) of *Pseudokirchneriella subcapitata* exposed for 72 hours to HMPA

Test parameters	Control	HMPA [mg/L]				
	-	7.3	14	29	60	115
Mean number of algae cells after 72 hours [x 1000 Cells/mL]	106.5	108.9	96.4	89.1	81.2	65.8
Cell growth rate reduction (0-72 h) [%]		-1	0	1	2	8
Cell growth inhibition (0-72 h) [%]		-7	1	4	9	38

After 72 hours of exposure, inhibition of cell density in the 7.3, 14, 29, 60, and 115 mg HMPA/L treatment groups was -7, 1, 4, 9, and 38%, respectively, relative to the negative control. Inhibition of growth rate in the 7.3, 14, 29, 60, and 115 mg HMPA/L treatment groups was -1, 0, 1, 2, and 8%, respectively, relative to the negative control. Dunnett's test indicated that cell density and growth rate were significantly reduced ($\alpha = 0.05$) in the 115 mg HMPA/L treatment level relative to the negative control. Consequently, the 72-hour NOAEC determined by this study was 60 mg HMPA/L.

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The biomass in the control cultures increased by a factor of >16 (299 in the test), the coefficient of variance for section specific growth rates was ≤35% (23.4% in the test), for the whole test period it was ≤7% (0.96% in the test). All validity criteria according to guideline OECD 201 are therefore fulfilled.

III. CONCLUSION

The 72 h E_rC_{50} and E_YC_{50} toxicity values for *P. subcapitata* exposed to HMPA were both determined to be >115 mg HMPA/L. The NOEC was determined to be 60 mg HMPA/L.

IIA 8.5 Effects on sediment dwelling organisms

In the water-sediment study for glyphosate, greater than 10% of the applied parent radiolabelled glyphosate was found in the sediment at Day 14 (Annex IIA 7.2.1; Annex IIIA 9.2). This indicates that there is a potential for exposure to glyphosate for sediment-dwelling organisms. However, the NOEC values from the chronic *Daphnia* test for glyphosate acid are well above 0.1 mg/L indicating low toxicity to aquatic invertebrates. According to the Guidance Document on Aquatic Ecotoxicology 9SANCO/3268/2001 rev.4), specific toxicity studies on sediment-dwellers should therefore not be necessary.

IIA 8.6 Aquatic plants

A summary of all available relevant and compliant data (including data already reviewed during the 2001 EU evaluation of glyphosate) for glyphosate, glyphosate salts and the aquatic metabolites AMPA and HMPA is presented in Table 8.6-1.

Table 8.6-1: Toxicity of glyphosate acid, glyphosate IPA salt, and its metabolites AMPA and HMPA to aquatic macrophytes

Species	Test design	EC_{50} (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid				
Lemna gibba	14 d semi-static	**$EC_{50, frond count}$ = 12** $EC_{50, dry weight}$ = 20	AII 8.6/01 █5662/B ████ 1996/yes	-
Lemna gibba	14 d static	$EC_{50, frond count}$ = 25.5	1092-02-1100-5 ████ 1987/no	98-00103
Myriophyllum aquaticum	14 d static	$EC_{50, shoot length, relative increase}$ = 278.7 $EC_{50, shoot length, growth rate}$ = 276 $EC_{50, fresh weight, relative increase}$ = **12.3** $EC_{50, fresh weight, growth rate}$ = 23.4 $EC_{50, dry weight, relative increase}$ = 25.2 $EC_{50, dry weight, growth rate}$ = 18.0 $EC_{50, root length, relative increase}$ = 18.0 $EC_{50, root length, growth rate}$ > 500	AII 8.6/04 ██-015/4-80/A ████ 2012/yes	-
Glyphosate-IPA salt				

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Species	Test design	EC_{50} (mg a.s./L)	Reference/GLP	2001 EU evaluation monograph reference
Lemna gibba	14 d static	**$EC_{50, \text{frond count}}$ = 53.56** $EC_{50, \text{dry weight}}$ = 62.59	AII 8.6/02 980909 1999/yes	-
Lemna minor	7 d semi-static	$EC_{50, \text{frond count}}$ = 25.5 $EC_{50, \text{dry weight}}$ = 46.2 $EC_{50, \text{growth rate}}$ = 42.6	IIA 8.6/03 -1873 2002/yes	-
AMPA				
Myriophyllum aquaticum	14 d static	$EC_{50, \text{shoot length, relative increase}}$ = 103.3 $EC_{50, \text{shoot length, growth rate}}$ > 94.6 $EC_{50, \text{fresh weight, relative increase}}$ = **70.8** $EC_{50, \text{fresh weight, growth rate}}$ = 97.3 $EC_{50, \text{dry weight, relative increase}}$ = 63.2 $EC_{50, \text{dry weight, growth rate}}$ = 72.0 $EC_{50, \text{root length, relative increase}}$ = 31.1 $EC_{50, \text{root length, growth rate}}$ = 150.1	AII 8.6/05 -022/4-80/A 2012/yes	-
HMPA				
Lemna gibba	7 d semi-static	$EC_{50, \text{frond count}}$ >123 $EC_{50, \text{dry weight}}$ >123	AII 8.6/06 139A-397 2011/yes	-

Values in bold: Confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

For aquatic macrophytes, two studies conducted with *Lemna gibba* (1092-02-1100-5, , 1987 and 5662/B, 1996) were included in the previous 2001 EU-Evaluation of Glyphosate.

Although the study of (1987) was conducted pre-GLP, all validity criteria according to the current OECD guideline 221 are fulfilled. Studies not considered in the 2001 EU Evaluation of Glyphosate are summarised below, providing additional information on the ecotoxicological profile of glyphosate.

Annex point	Author(s)	Year	Study title
AII 8.6/01		1996	GLYPHOSATE ACID: Toxicity to duckweed (*Lemna gibba*) Report No: 5662/B Date: 1996-01-31 GLP: yes not published

Guideline: EPA FIFRA Subdivision J Guideline 123-2

Deviations to OECD 221: None

Dates of experimental work: 1996-01-17 to 1996-01-31

Executive Summary

The toxicity of Glyphosate acid on growth of *Lemna gibba* was evaluated in a 14 day semi-static (media renewal after 5 and 9 days) toxicity test, performed at concentrations of 0.75, 1.5, 3.0, 6.0, 12, 24, 48 and 96 mg test item/L. A negative control (Hoaglands M media only) was prepared in parallel.

Three replicate vessels were prepared for the control and each test group. Three plants were added to each replicate (four fronds per plant). Test vessels were 400 mL beakers each containing 160 mL of control or test medium.

Frond numbers were determined in all vessels after 2, 5, 7, 9, 12 and 14 days. Toxicity symptoms were also recorded. Test media were analysed for Glyphosate acid content on day 0, 5 and 9 (fresh media) and on days 5, 9 and 14 (old media). The measured concentrations in the fresh media ranged from 90 – 108% of nominal and in the old media from 87 – 102% of nominal. The overall mean measured concentrations were 93 – 100% of nominal. Glyphosate acid was not detected in the control group.

Result showed a significant inhibition of frond number growth of *Lemna gibba* at nominal concentrations of 6.00 test item/L and higher, with significant tissue weight inhibition at 3.00 mg test item/L and higher. All validity criteria according to OECD 221 were fulfilled.

Glyphosate acid significantly inhibited the growth of *Lemna gibba* after 14 days at or above a nominal concentration of 6 mg test item/L. The 14-d EC_{50} value for inhibition of front number was 12 mg glyphosate acid/L (95% CL= 11- 14). The 14-d EC_{50} value for inhibition of tissue dry weight was 20 mg glyphosate acid/L (95% CL= 18 – 22). The NOEC values were determined to be 3.0 and 6.0 mg a.s./L for frond number and tissue dry weight increase, respectively.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6%
2. Vehicle and/or positive control:	Hoaglands M medium
3. Test organism:	
Species:	*Lemna gibba*, Strain G3
Source:	[redacted] Canada
4. Environmental conditions:	
Temperature:	24.6 – 25.0°C
Photoperiod:	24 h illumination
Light intensity	5000 lux
pH:	Freshly prepared test media: Control:4.7 – 4.9 0.75 mg/L: 4.7 – 4.8 1.5 mg/: 4.6 – 4.7 3.0 mg/L: 4.6

6.0 mg/L: 4.5
12 mg/L: 4.4
24 mg/L: 4.2 – 4.3
48 mg/L: 3.9 – 4.0
96 mg/: 3.5 – 3.6

Old test media:

Control:5.3 – 5.7
0.75 mg/L: 5.3 – 5.8
1.5 mg/: 5.2 – 5.8
3.0 mg/L: 5.2 – 5.8
6.0 mg/L: 5.1 – 5.7
12 mg/L: 4.8 – 5.6
24 mg/L: 4.6 – 5.0
48 mg/L: 4.0 – 4.2
96 mg/: 3.6 – 3.7

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity test on *Lemna gibba* was performed with eight concentration levels, 0.75, 1.5, 3.0, 6.0, 12, 24, 48 and 96 mg glyphosate acid/L with 3 replicates per test concentration. Three control replicates (without test substance) were tested under the same conditions as the test groups.

The plants were placed in 400 mL beakers (test vessels), containing 160 mL of Hoagland's M-medium prepared according to █████ (1961). The test was conducted under semi-static conditions with renewal of the test medium after 5 and 9 days. Three uniform healthy-looking plants with 4 fronds each were added to each control and test vessel.

2. Observations: The number of plants and fronds were counted after 2, 5, 7, 9, 12 and 14 days. Also symptoms of toxicity were recorded on these dates. At test end the weight of the dried plant tissue (at 60 °C) was recorded. The pH was measured in the old and the new test medium (new= day 0, 5 and 9, old = day 5, 9 and 14). Temperature in the test chamber was recorded daily and light intensity was recorded once a week.

Analytical measurements of glyphosate acid were performed by means of HPLC analysis at test start and after 5 and 9 d (after test medium renewal). Fresh media was analysed on days 0, 5 and 9. Old media were analysed on days 5, 9 and 14.

3. Statistical calculations: The EC_{50} and its 95% confidence interval were calculated by moving average angle method. The NOEC values were determined by calculation of statistical significance using one-way analysis of variance (ANOVA) and Dunnett's test for inhibition of frond number and biomass dry weight, respectively, at p = 0.05.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Analytical measurements were performed in the freshly prepared (day 0, 5 and 9) and the old (day 5, 9 and 14) test media. The measured concentrations in the fresh media ranged from 90 – 108% of nominal and in the old media from 87 – 102% of nominal (overall mean measured: 93 – 100% of nominal). As the mean measured content of the test item always ranged between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The EC_{50}, NOEC and LOEC values for glyphosate acid are given below based on nominal concentrations.

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Endpoint	Frond number [mg a.s./L]	Biomass dry weight [mg a.s./L]	Visual observed effects [mg a.s./L]
EC_{50} (95% CL)	12 (11 – 14)	20 (18 – 20)	-
NOEC	3.0	6.0	1.5
LOEC	6.0	12	-

B. OBSERVATIONS

The increase in frond number was significantly inhibited at nominal test concentration of 6.0 mg test item/L and higher, when compared to the control. The growth of the plant in terms of tissue dry weight was significantly reduced at 12 mg test item/L and higher. At 24, 48 and 96 mg test item/L dose related symptoms like pale frond colouration, emergence of stunted new frond growth, reduced root growth and unnatural floating on the solution surface were observed from day 2 onwards. Visually observed effects were apparent at concentrations of 3.0 mg/L and above. Therefore, the overall NOEC (empirically determined) was 1.5 mg a.s./L.

Table 8.6-2: Frond numbers, increase in frond numbers and inhibition compared to the control

Test item rate [mg a.s./L]	Number of fronds						Increase in frond numbers	Inhibition [%]
	Day 2	Day 5	Day 7	Day 9	Day 12	Day 14	(Day 0 – 14)	
Control	21	48	85	134	222	327	315	-
0.75	23	47	79	125	232	343	331	0
1.5	23	45	78	113	220	323	311	1
3.0	21	48	78	120	206	300	288	9
6.0	21	49	81	116	198	269	257	18*
12	20	44	74	105	148	173	161	49*
24	16	28	44	59	82	91	79	75*
48	15	21	24	28	28	30	18	94*
96	13	14	15	16	18	17	5	98*

* Significant inhibition compared to the control medium

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Table 8.6-3: Mean dry weight of plant tissue after 14 d, main increase in dry weight and inhibition compared to the control

Test item rate [mg a.s./L]	Mean tissue dry weight after 14 day [mg]	Mean increase [mg]	Inhibition [%]
Control	40.7	39.2	-
0.75	51.3	49.8	0
1.5	49.8	48.3	0
3.0	44.0	42.5	0
6.0	40.3	38.8	1
12	29.8	28.3	28*
24	16.5	15.0	62*
48	6.0	4.5	89*
96	1.4	< 0.1	100*

* Significant inhibition compared to the control medium

All validity criteria according to OECD 221 were fulfilled, as the doubling time of frond numbers in the control were less than 2.4/d.

III. CONCLUSION

Glyphosate acid was found to significantly inhibit the growth of *Lemna gibba* after 14 days at or above a nominal concentration of 6 mg a.s./L. The 14-d EC_{50} value for inhibition of front number was 12 mg a.s./L (95% CL= 11- 14 mg a.s./L) and for tissue dry weight 20 mg a.s./L (95% CL= 18 – 22mg a.s./L).The NOEC was determined to be 3.0 and 6.0 mg a.s./L for frond number and weight increase, respectively.

Annex point	Author(s)	Year	Study title
AII 8.6/02	███	1999	Glyphosate 62% IPA-Salt, Aquatic Plant toxicity Test using *Lemna gibba* ███ Report No: 980909█ Date: 1999-02-12 GLP: yes not published

Guideline: Guideline ASTM E 1415- 91 (June 1991)

Deviations to from OECD 221: None

Dates of experimental work: December 07, 1998 – December 21, 1998

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Executive Summary

The effects of glyphosate isopropylamine salt on growth of *Lemna gibba* were evaluated in a 14 day semi-static (media renewal on day 2, 4, 7, 9 and 11) toxicity test with five concentration levels, 6.25, 12.5, 25, 50 and 100 mg glyphosate IPA salt/L, equivalent to 3.9, 7.8, 15.6, 31.2 and 62.4 mg glyphosate acid/L and a negative control (culture medium only) was prepared in parallel. Three 3 replicates were prepared for each control or test item treatment using three plants per replicate (four fronds per plant). The number of

fronds affected was determined on day 0, 7 and 14. Observation of change in colour, break-up of plants and destruction of roots was conducted on day 7 and 14. Dry biomass weight was determined on day 14 (end of the test).

All test concentrations and control replicates were analysed on day 4 and day 11 (freshly prepared media) and on day 7 and 14 (3 day old test media). Glyphosate isopropylamine salt concentrations were measured as glyphosate acid on days 4 and 11 (fresh media) and on days 7 and 14 (old media). Glyphosate isopropylamine salt was not detected in the control group. Mean measured concentrations ranged from 80 to 120% of nominal concentrations.

Result showed an increase of growth of *Lemna gibba* at nominal concentrations of 6.25, 12.5 and 25 mg test item/L. Glyphosate isopropylamine salt was found to significantly inhibit the growth of *Lemna gibba* after 14 days at or above concentrations of 50 mg test item/L. The validity criteria according to guideline OECD 221 are fulfilled.

The EC_{50} values for inhibition of front number and dry weight after 14 days were 53.56 mg glyphosate IPA salt/L (equivalent to 33.42 mg glyphosate acid/L) and 62.59 mg glyphosate IPA salt/L (equivalent to 39.06 mg glyphosate acid/L) respectively. The NOEC was determined to be 25 mg glyphosate IPA salt/L equivalent to 15.60 mg glyphosate acid/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate 62% IPA-Salt
name	Glyphosate Isopropylamine Salt
Description:	Clear, liquid, yellowish
Lot/Batch #:	22-9754
Purity:	62.4% glyphosate IPA-Salt
Density:	1.2355 g/mL

2. Vehicle and/or positive control: 20X-AAP media , zinc chloride

3. Test organism:

Species:	*Lemna gibba*
Source:	[redacted] Germany

4. Environmental conditions:

Temperature:	23.9 – 26.9 °C
Photoperiod:	24 h fluorescence light
Light intensity	from 4941 – 5629 lux
pH:	7.40 – 7.56 in new test media, 7.81-9.08 in old test media
Conductivity:	not stated
Hardness:	not stated

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of a range finding test, the definitive test was performed with five concentration levels, 6.25, 12.5, 25, 50 and 100 mg test item/L with 3 replicates per test concentration. Three negative control replicates (20X-AAP media only) were tested under the same conditions. Three plants per replicate were used.

The plants were placed in 500 mL beakers (test vessels), which already contained 300 mL 20X-AAP dilution media prepared according to the guideline. The pH of the test medium was adjusted to 7.5 ±1 prior to the test. Three uniformly healthy-looking plants with 4 fronds each were used in each test vessel.

The test was conducted under semi-static conditions with renewal of test media on day 2, 4, 7, 9 and 11. Glyphosate isopropylamine salt concentrations were measured via HPLC as glyphosate acid on days 4 and 11 (fresh media) and on days 7 and 14 (old media).

The reference substance (zinc chloride) was equally tested from December 7 to 14, 1998 at 1.0, 3.2 and 10 mg/L resulting in 7 d E_bC_{50} of 4.67 mg/L and E_rC_{50} of 5.47 mg/L.

2. Observations:

Biological data: The number of plants and fronds in all test vessels was determined on day 0, 7 and 14. Observation of change in colour, break-up of plants and destruction of roots were made on day 7 and 14. Dry biomass weight was determined on day 14.

Physical data: The pH values were measured on day 0, 2, 4, 7, 9, 11 and 14. The room temperature in the test chamber was measured and recorded continuously. Sampling and analysis of the test concentration were carried out on day 4 and day 11 (freshly prepared media) and on day 7 and 14 (3 day old test media). All test concentrations and control replicates were analysed.

3. Statistical calculations: EC_{50} and EC_{90} values of frond number inhibition after day 7 and 14 were calculated by Probit analysis. The NOEC values were determined by calculation of statistical significance using one-way analysis of variance (ANOVA) and Dunnett's test for inhibition of frond number and biomass dray weight, respectively, at $\alpha = 0.05$.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: In freshly prepared test media the mean recoveries of the active substance varied between 81% and 85% for day 4 and107% to 111% for day 11. In the aged test media (3 days old),97% to 109% of the active substance were recovered for day 7 and 89% to103% for day 14. No active substance was found in the control samples.

As the mean measured content of the test item always ranged between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The EC_{50}, EC_{90} and NOEC values are given below based on nominal concentrations.

Endpoint	Glyphosate IPA salt [mg/L]	Glyphosate acid [mg/L]
$EC_{50,\ frond\ number}$ (7 day)	56.26 (C.I.: 45.53 – 69.53)	35.11 (C.I.: 28.41 – 43.39)
$NOEC_{frond\ number}$ (7 day)	25	15.60
$EC_{50,\ frond\ number}$ (14 day)	53.56 (C.I.: 42.91 - 66.85)	33.42 (C.I.: 26.78 - 41.71)
$NOEC_{frond\ number}$ (14 day)	25	15.60
$EC_{50,\ biomass}$ (14 day)	62.59 (C.I.: 47.94 - 81.73)	39.06 (C.I.: 29.91.- 51.00)

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NOEC $_{biomass}$ (14 day)	25	15.60

B. OBSERVATIONS

Observations: Increase of growth was found at nominal concentrations of 6.25, 12.5 and 25 mg test item/L. Glyphosate isopropylamine salt was found to significantly inhibit the growth of *Lemna gibba* after 14 days at or above a concentration of 50 mg test item/L. Front number inhibition values after day 14 as well as biomass dry weight inhibition are presented in the tables below.

Table 8.6-4: Frond numbers and inhibition values of *Lemna gibba* exposed to glyphosate IPA salt

		Control	Test item [mg/L]				
Glyphosate IPA			**6.25**	**12.5**	**25**	**50**	**100**
Glyphosate acid			**3.90**	**7.80**	**15.60**	**31.20**	**62.40**
Mean frond number	Day 0	12.0	12.0	12.0	12.0	12.0	12.0
	Day 7	91.0	127.7	113.7	120.3	56.3	12.7
	Day 14	535.0	776.7	757.3	875.7	119.3	20.7
Mean increase of frond number	Day 7	79.0	115.7	101.7	108.3	44.3	0.7
Mean inhibition [%]		-	-46± 17.6	-29± 11.4	-37± 6.4	-44± 18.1	-99± 1.5
Mean increase of frond number	Day 14	523.0	764.7	745.3	863.7	107.3	8.7
Mean inhibition [%]		-	-46± 14.0	-43± 12.7	-65± 15.4	79± 7.5	98± 1.1

Table 8.6-5: Dry weight after 14 days and inhibition values of *Lemna gibba* exposed to glyphosate IPA salt

		Control	Test item [mg/L]				
Glyphosate IPA			**6.25**	**12.5**	**25**	**50**	**100**
Glyphosate acid			**3.90**	**7.80**	**15.60**	**31.20**	**62.40**
Mean biomass dry weight [mg]	Day 14	48.9	65.2	66.0	69.8	18.7	6.6
Mean inhibition [%]		-	-33± 10.8	-35± 9.8	-43± 12.8	62± 15.4	86± 2.7

Visual observations: After 7 days smaller roots and fronds partially with more pigmentation were observed at 6.25, 12.5 and 25 mg test item/L. Smaller fronds and roots as well as fronds partially without pigmentation were observed at 50 and 100 mg glyphosate IPA/L. After 14 days fronds partially without pigmentation were observed at 12.5 and 25 mg glyphosate IPA/L. Smaller fronds and roots as well as fronds partially without pigmentation were observed at 50 and 100 mg test item/L.

The doubling time of frond numbers in the control was less than 2.5 days (60 h), corresponding to approximately a seven-fold increase in seven days. The validity criteria according to the current guideline OECD 221 are therefore fulfilled.

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III. CONCLUSION

Glyphosate isopropylamine salt was found to significantly inhibit the growth of *Lemna gibba* after 14 days at or above a nominal concentration of 50 mg a.s./L. The EC_{50} values for inhibition of front number and dry weight after 14 days were 53.56 mg glyphosate isopropylamine salt /L (equivalent to 33.42 mg glyphosate acid/L) and 62.59 mg glyphosate isopropylamine salt/L (equivalent to 39.06 mg glyphosate acid/L) respectively. The NOEC was determined to be 25 mg glyphosate isopropylamine salt/L, equivalent to 15.60mg glyphosate acid/L.

Annex point	Author(s)	Year	Study title
IIA 8.6/03	[redacted]	2002	IPA Salt of Glyphosate: Effects on *Lemna minor* [redacted] Report No: [redacted]-1873 Date: not stated GLP: yes Not published

Guideline: OECD Guideline 221 (2006)

Deviations to OECD 221: None

Dates of experimental work: Not stated

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Executive Summary

The effect of isopropylamine(IPA) salt of glyphosate on the growth of the duckweed *Lemna minor* was evaluated in a 7 day semi-static toxicity test at nominal concentrations of IPA salt of glyphosate of 2.92, 5.83, 11.7, 24.3, 48.6 and 97.2 mg IPA salt of glyphosate/L, equivalent to 2.16, 4.32, 8.64, 18.0, 36.0 and 72.0 mg glyphosate acid/L. Furthermore, a negative control group with *Lemna minor* exposed to test medium without test substance (negative control) was prepared in parallel.

The test vessels were 250mL glass beakers containing 150mL of the test or control medium. The vessels were continuously illuminated. The medium in each of the test vessels was renewed twice; day 2 and 4. Growth in each vessel was determined by counting the numbers of plants and fronds on three occasions during the definitive test and measuring the dry weights of the fronds after seven days. Analytical samples for analysis of glyphosate were collected from the three highest samples at the start and end of the test and following each media renewal (fresh and old media). Glyphosate isopropylamine salt was not detected in the control group. The mean measured content of the test item ranged between 96 and 104% of nominal, the results are therefore based on nominal concentrations.

Based on nominal concentrations of IPA salt of glyphosate, growth of *L. minor* was significantly inhibited at 24.3mg/L, but not affected at 11.7mg/L.

All validity criteria according to the OECD guideline 221 were fulfilled.

The lowest 7-day $EC_{50}$for *Lemna minor* exposed to glyphosate IPA salt was calculated to be 25.5 mg a.s./L, equivalent to 18.9 mg glyphosate acid/L. The 7-day NOEC for *Lemna minor* exposed to glyphosate IPA salt was determined to be 11.7 mg a.s./L, equivalent to 8.64 mg glyphosate acid/L. The lowest observed effect concentration (LOEC) of the IPA salt of glyphosate to *Lemna minor* measured over a 7 day exposure period was 24.3 mg glyphosate IPA salt/L, equivalent to 18.0 mg glyphosate acid/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine (IPA) salt
Description:	White powder
Lot/Batch #:	1002B
Purity:	97.1% as IPA salt
2. Vehicle and/or positive control:	Swedish Standard (SIS) *Lemna* growth medium
3. Test organism:	
Species:	*Lemna minor*
Source:	[redacted] UK
4. Environmental conditions:	
Temperature:	20.5 – 22.8°C
Photoperiod:	24 h fluorescence light
Light intensity	6600 - 8100 Lux
pH:	6.06 – 6.96

B. STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of a range finding test, the definitive test was performed with six concentration levels, 2.92, 5.83, 11.7, 48.6 and 97.2 mg glyphosate IPA salt/L, equivalent to 2.16, 4.32, 8.64, 18.0, 36.0 and 72.0 mg glyphosate/L. Furthermore, a negative control group with *Lemna minor* exposed to culture medium (SIS) only was run in parallel. The medium in each of the test vessels was renewed on day 2 and 4.Three replicates were prepared with 9-10 fronds (in 3-4 colonies) were used for each test concentration and control. Temperatures and pH values were measured in the test media were measured at the start of tests and at the end. In addition, temperature was monitored continuously. Analytical samples for analysis of glyphosate were collected at the start of the tests and at the end and following each media renewal. Samples were analysed using HPLC with fluorescence detection.

2. Observations: The numbers of fronds and colonies were counted on days 0 (start), 2, 4 and 7 during the definitive test. Dry weights of the fronds were determined at the end of the tests. The fronds from each vessel were collected, rinsed with de-ionised water and dried at 60°C to a constant weight. The dry weights of fronds from each vessel were measured to ±0.1 mg.

3. Statistical calculations: EC_{50} values were calculated using the LC_{50} program of Stephan *et al.*, 1986. The no-observed-effect concentration (NOEC) and the lowest- observed-effect concentration (LOEC) were based on statistical analysis of *L. minor* final frond numbers, growth rate and area under growth curve values, as well as the final biomass, for the definitive test. Data were first tested for compliance with the assumptions of ANOVA in terms of normality of distribution and homogeneity. The treatment means were tested for significant difference from the control mean at α=0.05 using the Dunnett's test.

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II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Chemical analyses were performed on samples of the test media to quantify glyphosate in the test solution. The mean measured content of the test item always ranged between 80 and 120% of nominal.

The endpoints given below are based on nominal concentrations of IPA salt of glyphosate and glyphosate acid.

Endpoint	Glyphosate IPA-salt [mg/L]	Glyphosate acid [mg/L]
$EC_{50, \text{frond number}}$ (7 day)	25.5 (C.I.: 11.1 – 73.4)	18.9 (C.I.: 8.2 – 54.4)
$NOEC_{\text{frond number}}$ (7 day)	11.7	8.64
$EC_{50, \text{biomass}}$ (7 day)	46.2 (C.I.: 18.6 – 1673)	34.2 (C.I.: 13.8 – 1239)
$NOEC_{\text{biomass}}$ (7 day)	11.7	8.64
$EC_{50, \text{area under growth curve}}$ (7 day)	Not calculable	Not calculable
$NOEC_{\text{area under growth curve}}$ (7 day)	11.7	8.64
$EC_{50, \text{growth rate}}$ (7 day)	42.6 (C.I.: 26.3 – 87.8)	31.6 (C.I.: 19.5 – 65.0)
$NOEC_{\text{growth rate}}$ (7 day)	11.7	8.64

B. OBSERVATIONS

The results of the definitive test showed no effect on frond growth at 11.7 mg glyphosate isopropylamine salt/L and partial and statistically significant inhibition at 24.3 mg/L. At 48.6 and 97.2 mg glyphosate IPA salt/L the inhibition of frond growth was greater at 81% and 87% inhibition for final frond numbers.

The validity criteria according to guideline OECD 221 are fulfilled.

III. CONCLUSION

The lowest observed effect concentration (LOEC) of the IPA salt of glyphosate to *Lemna minor* measured over a 7 day exposure period was 24.3 mg glyphosate IPA salt/L, equivalent to 18.0 mg glyphosate acid/L. The overall no-observed effect concentration (NOEC) of the IPA salt of glyphosate to *Lemna minor* measured over a 7 day exposure period was 11.7 mg/L, equivalent to 8.64 mg glyphosate acid/L. The lowest 7 day EC_{50} was 25.5 mg/L with 95% confidence limits of 11.1 to 73.4 mg glyphosate IPA salt/L measured from final frond numbers at 7 days, equivalent to 18.9 mg glyphosate acid/L (8.22 – 54.37 mg a.s./L).

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Annex point	Author(s)	Year	Study title
AII 8.6/04	[redacted]	2012	Effect of MON77973 (Glyphosate acid) on the Growth of *Myriophyllum aquaticum* in the Presence of Sediment. Test with a subsequent Recovery Period. [redacted] Report No [redacted]-015/4-80/A Date: 2012-02-21 GLP: yes not published

Guideline: Maltby, L., *et al.* (2008): Aquatic Macrophyte Risk Assessment for Pesticides, SETAC AMRAP

Deviations to guideline none

Dates of experimental work: 2010-09-27 to 2010-10-11

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Executive Summary

The toxicity of Glyphosate acid on growth of *Myriophyllum aquaticum* was evaluated in a 14 day static toxicity test, with subsequent 7 day recovery test, performed at concentrations of 5.0, 15.8, 50, 158 and 500 mg glyphosate/L, equivalent to 5.87, 18.5, 58.7, 185.4 and 587 mg glyphosate acid/L. A negative control (Smart & Bako medium) was prepared in parallel.

Two sets of vessels (exposure and recovery set) were prepared, with each set comprising three replicates for each test concentration and six replicates for the controls. Test vessels were 2-L beakers, each containing five individual plants potted in individual pots containing artificial sediment. Plant length, fresh weight, dry weight and root length were determined in all vessels. Plant length was recorded at test start and after 3, 7, 10 and 14 days and after 21 days (recovery vessels). At test start and test end, fresh weight of each plant was determined. Dry weight was determined at test initiation using 25 additional plants and at test end on the tested plants. At the end of the test all plants were harvested and the root length was assessed semi-quantitatively in terms of length of the main root. After 14 days, all plants in recovery vessels were transferred to vessels containing dilution water only to assess recovery following exposure.

Test media were analysed for Glyphosate acid content at test start and end of exposure and recovery periods. The measured concentrations ranged from 92.0 – 100.6% of nominal. Glyphosate acid was not detected in the control group.

Relative to the control group, at the highest treatment rate (500 mg glyphosate acid/L) there was 100% growth inhibition based on fresh weights, at the 5.0 mg Glyphosate acid/L. At 500 mg Glyphosate acid/L fresh weight increase was inhibited by 100%, shoot length increase by 70.8% and growth rate by 57.1%. The recovery period demonstrated that *Myriophyllum aquaticum* pre-exposed to up to 50.0 mg Glyphosate acid/L were able to recover to control levels of growth, in untreated culture medium within 7 days of transfer.

The study fulfilled the validity criteria of achieving at least 50% increase in control plant growth in terms of length within 7 days of test initiation. The test was therefore considered to be valid.

Glyphosate acid significantly inhibited the fresh weight of *Myriophyllum aquaticum* after 14 days at a nominal concentration of <5.0 mg glyphosate acid/L. Shoot length was inhibited at or above nominal concentrations of 5.0 mg glyphosate acid/L. The 14-d EC_{50} value for fresh weight inhibition was 12.3 mg glyphosate acid/L and for shoot length it was78.7 mg glyphosate acid/L. *Myriophyllum*

***aquaticum* pre-exposed for 14 day to up to 50.0 mg glyphosate acid/L were able to recover in untreated culture medium after a 7 day recovery period.**

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid (MON77973)
Description: White crystalline powder
Lot/Batch #: GLP-0807-19475-T
Purity: 85.2% Glyphosate

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Myriophyllum aquaticum*
Source: [redacted] Germany

4. Environmental conditions:

Growth medium: Smart & Bako medium
Artificial sediment: 4-5% peat
20% kaolin clay
75-76% quartz sand
$CaCO_3$ (if needed to adjust pH to 7.0 ± 0.5)
Based on artificial soil used in OECD guideline 219
Moistening of sediment up to 30% with deionised water or nutrient medium (ammonium chloride and sodium phosphate)
Temperature: 18.0-20.5 °C
Photoperiod: 16 h light/ 8 h dark
Light intensity 6541-7097 lux
pH: Values recorded at test start and end (in brackets) of 14 day exposure period:
Controls = 7.99 (8.14-9.06)
5 mg/L = 8.06 (8.77-10.0)
15.8 mg/L: = 7.99 (8.96-9.96)
50.0 mg/L = 7.36 (7.35-9.13)
158 mg/L = 3.84 (4.88-5.28)
500 mg/L = 2.80 (3.29-3.43)

Values at start and end of 7 day recovery period:
Recovery period start = 7.95
Recovery period end = 8.17 – 9.48

Oxygen saturation 14 day exposure period:
92 – 94% at the start of the test
114 – 193% at the end of the test

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7 day recovery period:

96% at the start of the test
95 – 131% at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity test on *Myriophyllum aquaticum* was performed with six concentration levels of 5.0, 15.8, 50, 158 and 500 mg glyphosate/L, equivalent to 5.87, 18.5, 58.7, 185.4 and 587 mg Glyphosate acid/L, with 3 replicates per test concentration. Six control replicates (without test substance) were tested under the same conditions as the test groups. Two sets of vessels (exposure and recovery) were prepared at the start of the test

The plants were planted in small plastic plant pots into sediment and placed in glass beakers (test vessels), containing 2 L Smart & Bako medium. The test was conducted under static conditions. Five plants were added to each test and control replicate.

After 14 days exposure plants in the recovery set of *Myriophyllum aquaticum* replicates, exposed to the same concentration levels, were transferred into freshly prepared test medium without test item to determine the potential recovery after an exposure event.

2. Observations: Plant length, fresh weight, dry weight and root length were determined in all vessels. Plant length was recorded at test start and after 3, 7, 10 and 14 days. At test start and test end, fresh weight of each plant was determined. Dry weight was determined at test initiation using 25 additional plants and at test end on the tested plants (dried at 105 °C for 24 h). At the end of the test all plants were harvested and the root length was assessed semi-quantitatively in terms of length of the main root. Temperature in the test chamber was recorded continuously. Oxygen content, pH and light intensity was at test start and after 14 days.

Analytical control measurements of the actual concentration of the glyphosate acid were performed by means of LC/MS-MS analysis at test start, after 14 (after exposure phase) and 21 days (after recovery phase).

3. Statistical calculations: The EC_{10}, EC_{20} and EC_{50} and its 95% confidence interval were calculated by Probit analysis modified for continuous data. The NOEC values were determined by calculation of statistical significance using one-way analysis of variance (ANOVA), followed by Williams' t-test, Dunnett's t-test or Welch's t-test ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Analytical control measurements of the actual concentration of the glyphosate acid were performed at test start, after 14 and 21 days (after recovery phase). The measured concentrations ranged from 92.0 – 100.6% of nominal. As the mean measured content of the test item always ranged between 80 and 120% of nominal, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The $EC_{50}$and NOEC values after 14 day growth inhibition test are given below based on nominal concentrations.

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Endpoint	Glyphosate acid [mg/L]	
	14 Day EC_{50}	14 Day NOEC
Shoot length/relative increase	78.7 (46.1- -146)	5.0
Shoot length/growth rate	276 (159-664)	<5.0
Fresh weight/relative increase	**12.3** (9.19-15.8)	<5.0
Fresh weight/ growth rate	23.4 (17.2-30.9)	<5.0
Dry weight/relative increase	25.2 (2.61-151)	50.0
Dry weight/ growth rate	18.0 (5.19-43.0)	50.0
Root length/relative increase	18.0 (5.19-43.0)	>5.0
Root length/growth rate	>500	<5.0

* CI = 95% confidence interval

The $EC_{50}$and NOEC values after 7 day recovery period are given below based on nominal concentrations.

Endpoint	Glyphosate acid [mg/L]	
	7 Day EC_{50}	7 Day NOEC
Shoot length/relative increase	99.5 (79.7-125)	50
Shoot length/growth rate	114 (89.5-147)	50
Fresh weight/relative increase	12.3	158
Fresh weight/ growth rate	n.d.	158
Dry weight/relative increase	n.d.	≥500
Dry weight/ growth rate	n.d.	≥500
Root length/relative increase	>500	≥500
Root length/growth rate	≥500	≥500

n.d.: not determined due to mathematical reasons or inappropriate data

B. OBSERVATIONS

There was a concentration dependent effect on growth, root length, fresh and dry weight of *Myriophyllum aquaticum.* Growth was significantly reduced at 5.00 mg glyphosate/L, fresh weight at <50 mg Glyphosate acid/L, dry weight at 50.0 mg Glyphosate acid/L and root length at <50 mg Glyphosate acid/L during the 14 day exposure test. In the subsequent recovery test it was shown that *Myriophyllum aquaticum*, pre-exposed to up to 50.0 mg Glyphosate acid/L were able to recover to control levels of growth in untreated culture medium within 7 days of the exposure period.

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Table 8.6-6: Percentage of inhibition of shoot length of *Myriophyllum aquaticum* exposed for 14 days to glyphosate acid

Test parameters	Glyphosate acid [mg/L]				
	5.0	15.8	50.0	158	500
Inhibition of shoot length increase (%)	19.2	29.9	55.9	50.3	70.8
Inhibition of shoot length growth rate (%)	11.8	19.5	41.9	36.7	57.9
Inhibition of fresh weight increase (%)	34.2	57.5	69.2	83.7	109
Inhibition of fresh weight growth rate (%)	24.6	46.5	59.0	76.7	115
Inhibition of dry weight increase (%)	-11.8	46.5	26.8	92.7	108
Inhibition of dry weight growth rate (%)	-10.2	40.8	40.4	92.4	114
Inhibition of root length increase (%)	19.4	52.3	76.0	79.7	88.8
Inhibition of root length growth rate (%)	2.0	7.0	13.5	15.1	21.1

The study fulfils the validity criteria as stated in the study plan which follows the criteria established by the AMRAP working group, with an increase of biomass (shoot length) in controls was > 50 %, indicating that continuous growth was supported throughout the test duration. Furthermore, constant maintenance of temperature (20 ± 2 °C) was also achieved.

III. CONCLUSION

Glyphosate acid significantly inhibited the fresh weight of *Myriophyllum aquaticum* after 14 days at a nominal concentration of <5.0 mg glyphosate acid/L. Shoot length was inhibited at or above nominal concentrations of 5.0 mg glyphosate acid/L. The 14-d EC_{50} value for fresh weight inhibition was 12.3 mg glyphosate acid/L and for shoot length it was78.7 mg glyphosate acid/L. *Myriophyllum aquaticum* pre-exposed for 14 day to up to 50.0 mg glyphosate acid/L were able to recover in untreated culture medium after a 7 day recovery period.

Annex point	Author(s)	Year	Study title
AII 8.6/05		2012	Effect of AMPA (Aminomethylphosphonic acid) on the Growth of *Myriophyllum aquaticum* in the Presence of Sediment, with a subsequent Recovery Period, including Amendment No 2 Report No: -022/4-80/A Date: 2012-02-12 GLP: yes not published

Guideline: Maltby, L., et al. (2008): Aquatic Macrophyte Risk Assessment for Pesticides, SETAC AMRAP

Dates of experimental work: 2011-08-18 to 2011-109-08

Executive Summary

The toxicity of Glyphosate acid on growth of *Myriophyllum aquaticum* was evaluated in a 14 day static toxicity test, with subsequent 7 day recovery test, performed at concentrations of 1.0, 2.6, 6.4, 16, 40 and 100 mg AMPA/L. A negative control (Smart & Bako medium) was prepared in parallel.

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Two sets of vessels (exposure and recovery set) were prepared, with each set comprising three replicates for each test concentration and six replicates for controls were used. Test vessels were 2-L beakers, each containing five individual plants potted in individual pots containing artificial sediment. Plant length, fresh weight, dry weight and root length were determined in all vessels. Plant length was recorded at test start and after 3, 7, 10 and 14 days and after 21 days (recovery vessels). At test start and test end, fresh weight of each plant was determined. Dry weight was determined at test initiation using 25 additional plants and at test end on the tested plants. At the end of the test all plants were harvested and the root length was assessed semi-quantitatively in terms of length of the main root. After 14 days, all plants in recovery vessels were transferred to vessels containing dilution water only to assess recovery following exposure.

Test media were analysed for AMPA content at test start, test end and at the end of the recovery period. The measured concentrations ranged from 75.5 - 102% of nominal. AMPA was not detected in the control group. Therefore the test was evaluated using the geometric mean measured concentrations.

Result showed a significant inhibition of fresh weight and shoot length at the lowest test concentration of >14.3 mg AMPA/L. The following recovery test demonstrated that *Myriophyllum aquaticum* pre-exposed to up to 5.4 mg AMPA/L were able to recover in untreated culture medium after a 7 day recovery period.

The study fulfilled the validity criteria of achieving at least 50% increase in control plant growth in terms of length within 7 days of test initiation. The test was therefore considered to be valid.

AMPA significantly inhibited the fresh weight and shoot length of *Myriophyllum aquaticum* after 14 days at a nominal concentration of >14.3 mg AMPA/L. The 14-d EC_{50} value for fresh weight inhibition was 70.8 mg AMPA/L and for shoot length > 94.6 mg AMPA/L. *Myriophyllum aquaticum* pre-exposed for 14 day to up to 5.4 mg AMPA/L were able to recover in untreated culture medium after a 7 day recovery period.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: AMPA (Aminomethylphosphonic acid)
Description: White crystalline solids
Lot/Batch #: GLP-0905-19864A (recertified as GLP-110521446-A)
Purity: 98.5%

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Myriophyllum aquaticum*
Source: [redacted] Germany

4. Environmental conditions:

Growth medium: Smart & Bako medium
Artificial sediment: 4-5% peat
20% kaolin clay
75-76% quartz sand
$CaCO_3$ (if needed to adjust pH to 7.0 ± 0.5)
Based on artificial soil used in OECD guideline 219

	Moistening of sediment up to 30% with deionised water or nutrient medium (ammonium chloride and sodium phosphate)
Temperature:	20.5 – 21.0 °C
Photoperiod:	16 h light/ 8 h dark
Light intensity	7571 - 7903 lux
pH:	Values recorded at test start and end (in brackets) of 14 day exposure period: Controls = 7.91 (8.54–8.91) 0.88 mg/L = 8.06 (8.04-8.08) 2.23 mg/L: = 7.99 (8.05-8.11) 5.43 mg/L = 7.36 (8.05-8.07) 14.3 mg/L = 3.84 (7.90-7.99) 37.1 mg/L = 2.80 (7.75-7.79) 94.6 mg/l = 6.60 (7.23-7.33) Values at start and end of 7 day recovery period: Recovery period start = 7.97-9.04 Recovery period end = 8.18 – 9.28
Oxygen saturation	14 day exposure period: 95 – 97% at the start of the test 101 – 138% at the end of the test 7 day recovery period: 96 – 138% at the start of the test 90 – 114% at the end of the test

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The toxicity test on *Myriophyllum aquaticum* was performed with six concentration levels of 1.0, 2.6, 6.4, 16, 40 and 100 mg AMPA/L with 3 replicates per test concentration. Six control replicates (without test substance) were tested under the same conditions as the test groups.

The plants were planted in small plastic plant pots into sediment and placed in glass beakers (test vessels), containing 2 L Smart & Bako medium. The test was conducted under static conditions. Five plants were added to each test and control replicate. After 14 days exposure another set of *Myriophyllum aquaticum* replicates, exposed to the same concentration levels, was transferred into freshly prepared test medium without test item to determine the potential recovery after an exposure event.

2. Observations: Plant length, fresh weight, dry weight and root length were determined in all vessels. Plant length was recorded at test start and after 5, 8 and 14 days. At test start and test end, fresh weight of each plant was determined. Dry weight was determined at test initiation using 25 additional plants and at test end on the tested plants (dried at 105 °C for 24 h). At the end of the test all plants were harvested and the root length was assessed semi-quantitatively in terms of length of the main root. Temperature in the test chamber was recorded continuously. Oxygen content, pH and light intensity was at test start and after 14 days.

Analytical control measurements of the actual concentration of AMPA were performed by means of LC/MS-MS analysis at test start, after 14 and 21 days (after recovery phase).

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3. Statistical calculations: The EC_{10}, EC_{20} and EC_{50} and its 95% confidence interval were calculated by Probit analysis modified for continuous data. The NOEC values were determined by calculation of statistical significance using one-way analysis of variance (ANOVA), followed by Dunnett's t-test or Welch's t-test (p = 0.05).

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Analytical control measurements of the actual concentration of AMPA were performed at test start, after 14 and 21 days (after recovery phase). The measured concentrations ranged from 75.5 102% of nominal. Therefore the test was evaluated using the geometric mean measured concentrations.

Measured concentrations of AMPA in the macrophyte growth inhibition test are depicted below.

Nominal [mg/L]	Test start 14 d growth test		End of test 14 d growth test		Mean measured [mg/L]
	Measured [mg/L]	% of nominal	Measured [mg/L]	% of nominal	
Control	< LOQ -	-	<LOQ	-	< LOQ
1.0	1.02	101.7	0.76	76.4	0.88
2.6	2.49	95.8	1.99	76.6	2.23
6.4	6.09	95.2	4.85	75.7	5.43
16	15.5	96.6	13.2	82.2	14.26
40	40.0	100.0	34.4	86.1	37.13
100	98.3	98.3	91.1	91.1	94.61

LOQ = limit of quantification = 0.5 mg/L

The EC_{50} and NOEC values after 14-day growth inhibition test are given below based on geometric mean measured concentrations.

Endpoint	AMPA [mg/L][#]	
	14 Day EC_{50}	14 Day NOEC
Shoot length/relative increase	103.3 (54.8-337)	14.3
Shoot length/growth rate	> 94.6	14.3
Fresh weight/relative increase	**70.8** (59.4-87.7)	14.3
Fresh weight/ growth rate	97.3 (81.8-126)	14.3
Dry weight/relative increase	63.2 (49.0-79.2)	37.1
Dry weight/ growth rate	72.0 (59.4-83.6)	37.1
Root length/relative increase	31.1 (28.1-34.6)	5.4
Root length/growth rate	150.1*(136.1-168.1)	5.4

* extrapolated, highest test concentration was 94.6 mg AMPA/L
[#] 95% confidence intervals presented in brackets.

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The EC_{50} and NOEC values after 7 day recovery period are given below based on geometric mean measured concentrations.

Endpoint	AMPA [mg/L][#]	
	7 Day EC_{50}	7 Day NOEC
Shoot length/relative increase	78.2 (34.2-6082.1)*	37.1
Shoot length/growth rate	92.8 (41.9-8310.6)	37.1
Fresh weight/relative increase	**12.6** (2.5-79.7)	5.4
Fresh weight/ growth rate	13.6 (2.8-87.3)	5.4
Dry weight/relative increase	≥ n.d.	≥ 94.6
Dry weight/ growth rate	n.d.	≥ 94.6
Root length/relative increase	≥ n.d.	≥ 94.6
Root length/growth rate	n.d.	≥ 94.6

[#] 95% confidence intervals presented in brackets.
n.d.: not determined due to mathematical reasons or inappropriate data

B. OBSERVATIONS

There was a concentration dependent effect on growth, fresh and dry weight of *Myriophyllum aquaticum.* Growth and fresh weight was significantly reduced at >14.3 mg AMPA/L. In the subsequent recovery test it was shown that *Myriophyllum aquaticum*, pre-exposed to up to 5.4 mg AMPA/L were able to recover in untreated culture medium after a 7 day recovery period.

Table 8.6-7: Percentage of inhibition of shoot length of *Myriophyllum aquaticum* exposed for 14 days to AMPA

Test parameters	AMPA [mg/L]					
	0.88	2.23	5.43	14.26	37.13	94.61
Inhibition of shoot length increase (%)	20.8	16.8	12.5	16.7	40.8	54.3
Inhibition of shoot length growth rate (%)	11.7	9.2	6.4	9.0	26.4	38.0
Inhibition of fresh weight increase (%)	-14.1	-15.2	-7.0	-10.9	29.0	60.2
Inhibition of fresh weight growth rate (%)	-9.0	-9.4	-3.9	-6.9	20.8	48.3
Inhibition of dry weight increase (%)	-47.5	-45.6	-7.1	1.1	-4.6	79.9
Inhibition of dry weight growth rate (%)	-28.9	-26.5	-4.9	1.6	-2.1	71.2
Inhibition of root length increase (%)	-13.1	-8.8	15.7	26.4	55.0	79.3
Inhibition of root length growth rate (%)	-3.5	-2.5	4.2	7.7	20.4	39.5

The study fulfils the validity criteria as stated in the study plan which follows the criteria established by the AMRAP working group; with an increase of biomass (shoot length) in controls was > 50 %, indicating that continuous growth was supported throughout the test duration. Furthermore, constant maintenance of temperature (20 ± 2 °C) was also achieved.

III. CONCLUSION

AMPA significantly inhibited the fresh weight and shoot length of *Myriophyllum aquaticum* after 14 days at a nominal concentration of >14.3 mg AMPA/L. The 14-d EC_{50} value for fresh weight inhibition was 70.8 mg AMPA/L and for shoot length > 94.6 mg AMPA/L. *Myriophyllum aquaticum*

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pre-exposed for 14 day to up to 5.4 mg AMPA/L were able to recover in untreated culture medium after a 7 day recovery period.

Annex point	Author(s)	Year	Study title
AII 8.6/06	[redacted]	2011	HMPA (hydroxymethylphosphonic acid): A 7-Day Static-Renewal Toxicity Test with Duckweed (*Lemna gibba* G3) [redacted] Report No: 139A-397 Date: 2011-10-11 GLP: yes not published

Guideline: OPPTS 850.4400
ASTM Standard Guide 1415-91 E (1991)
OECD Guideline 221 (2006)

Deviations from OECD 221: None

Dates of experimental work: 2010-06-10 to 2010-06-19

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Executive Summary

The effects of HMPA (hydroxymethylphosphonic acid) on growth of *Lemna gibba* G3 were evaluated in a 7-day static-renewal toxicity test at nominal concentrations of 7.5, 15, 30, 60, and 120 mg HMPA/L, corresponding to mean measured concentrations of 7.4, 15, 30, 60 and 123 mg HMPA/L, respectively. A negative control was prepared in parallel. Three replicates were prepared per control and test item treatment using four plants (totalling 12 fronds) per replicate, each. The pH of the 20X AAP test medium was adjusted to 7.6 with 0.1 N NaOH. Renewal of the test media was performed on day 3 after test initiation. Direct counts of number of fronds were conducted on day 3, 5 and 7. Observations of chlorosis, necrosis, break-up of duckweed colonies, root destruction, death and any other abnormalities in plant or frond appearance were also performed at those times. Dry weight was determined at the beginning (representative sample) and at the end of the test (each vessel). EC_{50} values were calculated based on replicate frond counts, biomass and growth rates based on frond counts and biomass on day 7 of the test. Analysis of the test concentration was carried out at test initiation, on day 3 and at test termination on day 7. The mean measured content of the test item ranged between 99 and 103% of nominal concentrations. HMPA was not detected in the control group.

Percent inhibition of frond growth in the 7.4, 15, 30, 60 and 123 mg HMPA/L treatment groups at test termination was -9, -15, -1, -7 and -20%, respectively. Percent inhibition of growth rate based on frond number in the 7.4, 15, 30, 60 and 123 mg HMPA/L treatment groups at test termination was -4, -6, -1, -4, and -8%, respectively. Percent inhibition biomass in the 7.4, 15, 30, 60 and 123 mg HMPA/L treatment groups at test termination was -13, -25, -15, -20 and -33%, respectively. Percent inhibition of growth rate based on biomass in the 7.4, 15, 30, 60 and 123 mg HMPA/L treatment groups at test termination was -5, -9, -6, -8 and -12%, respectively.

Based on these results, the EC_{50} for frond number, biomass and growth rates based on frond number and biomass for HMPA was determined to be >123 mg HMPA/L. After 7 days of exposure, there were no apparent treatment-related effects upon growth at any of the concentrations tested. The validity criteria according to guideline OECD 221 are fulfilled.

Since no inhibition effects of HMPA were observed on frond number, frond number growth rate, biomass and biomass growth rate of *Lemna gibba* after 7 days at all concentrations tested, the EC_{50}

values after 7 days of exposure were all >123 mg HMPA/L, the highest concentration tested. The NOEC was determined to be 123 mg HMPA/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	HMPA (hydroxymethylphosphonic acid)
Description:	Solid
Lot/Batch #:	GLP-1003-20448-A
Purity:	97.0%.
2. Vehicle and/or positive control:	20X AAP medium
3. Test organism:	
Species:	*Lemna gibba* G3, up to 7 days old
Source:	[redacted]
4. Environmental conditions:	
Temperature:	23.7 – 25.4 °C
Light intensity:	Continuous illumination, 4410 – 5250 lux
pH:	7.1 – 8.0 at test start; 8.8 – 9.0 at test termination
Hardness:	20.88 mg (K_2HPO_4/L)

B: STUDY DESIGN AND METHODS

1. Experimental treatments: On the basis of the results of a range finding test, the definitive test was performed at five concentration levels, 7.5, 15, 30, 60, and 120 mg HMPA/L with 3 replicates per test concentration. Three control replicates (without test substance) were tested under the same conditions. Four plants totalling 12 fronds were added to each replicate test chamber. The plants were placed in 250 mL test vessels containing 100 mL 20X-AAP test media. The pH of the test medium was adjusted with 0.1N NaOH prior to the test. The test was conducted under a 7-day static-renewal test conditions. The renewal of the test media was performed on day 3 after test initiation.

2. Observations:

Biological data: The toxicity of HMPA to duckweed was determined by direct counts of frond numbers and observations for chlorosis, necrosis, dead fronds and frond appearance were made on Days 3, 5 and 7. Dry weight was measured at the beginning of the test on a representative sample from the culture used to initiate the test. At the end of the test, dry weight was determined from each test vessel.

Physical data: The pH values were measured on day 0, 3, and 7. Temperature was measured continuously and recorded twice daily. Samples of the test solutions were collected from new solution of each experimental group at the beginning of the test, from new solutions and pooled old solutions at the end of the renewal period on Day 3, and from pooled test solutions at test termination to determine test substance concentrations. Samples were processed immediately for analysis. All test concentrations and control replicates were analysed using HPLC with mass selective detection.

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3. Statistical calculations: The 7-day EC_{50} value for frond counts; biomass and growth rates based on frond counts and biomass are based on descriptive analysis of the data. The NOEC values were determined by calculation of statistical significance using one-way analysis of variance (ANOVA) and Dunnett's test for inhibition of frond number and biomass dry weight, respectively, at $\alpha = 0.05$.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: In freshly prepared test media the recovery of the active substance ranged between 92.5 % and 103%. In the aged test media (7 days old), 104% to 110% of the active substance was recovered. Samples from new and old test solution at Day 3 renewal ranged from 90.1 to 101% and 96.9 to 107%, respectively.

The EC_{50} and NOEC values are given below based on mean measured concentrations.

Endpoint	mg HMPA/L
$EC_{50, \text{frond number}}$ (7 day)	>123
$NOEC_{\text{frond number}}$ (7 day)	123
$EC_{50, \text{biomass}}$ (7 day)	>123
$NOEC_{\text{biomass}}$ (7 day)	123
$EC_{50, \text{growth rate (frond number)}}$ (7 day)	>123
$NOEC_{\text{growth rate (frond number)}}$ (7 day)	123
$EC_{50, \text{growth rate (biomass)}}$ (7 day)	>123
$NOEC_{\text{growth rate (biomass)}}$(7 day)	123

B. OBSERVATIONS

Observations: None of the parameters recorded, i.e. frond number, biomass, growth rate based on front number and growth rate based on biomass was found to be significantly different from the control (Dunnett's t-test ($\alpha = 0.05$) see Table 8.6-8..

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Table 8.6-8: Frond numbers and inhibition values of *Lemna gibba* G3 after 7 days of exposure to HMPA

Test item	Control	HMPA [mg/L]				
Nominal concentrations [mg HMPA/L]	**-**	7.5	15	30	60	120
Mean measured concentrations [mg HMPA/L]	**-**	7.4	15	30	60	123
Mean frond number	145	158	166	147	156	174
Mean inhibition [%]	-	-9	-15	-1	-7	-20
Mean biomass [mg]	16.73	18.90	20.93	19.17	20.10	22.20
Mean inhibition [%]		-13	-25	-15	-20	-33
Mean growth rate based on frond number	0.3531	0.3681	0.3751	0.3564	0.3656	0.3818
Mean inhibition [%]	-	-4	-6	-1	-4	-8
Mean growth rate based on biomass	0.3494	0.3679	0.3821	0.3699	0.3763	0.3909
Mean inhibition [%]	-	-5	-9	-6	-8	-12

The doubling time of frond numbers in the control was less than 2,5 days (1.96 days), corresponding to approximately a twelve-fold increase after seven days. The validity criteria according to the current guideline OECD 221 are therefore fulfilled.

III. CONCLUSION

Since no inhibition effects of HMPA was observed on the frond number, frond number growth rate, biomass and biomass growth rate of *Lemna gibba* G3 after 7 days at all concentrations tested, the EC_{50} values for frond number, frond number growth rate, biomass and biomass growth rate were all >123 mg HMPA/L, the highest concentration tested. The NOEC was determined to be 123 mg HMPA/L.

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IIA 8.7 Effects on Bees

There is an extensive regulatory database assessing the acute contact and oral toxicity of glyphosate and glyphosate salts. The results from all of these studies demonstrate that glyphosate and glyphosate salts have low acute contact and oral toxicity to honeybees. The 2001 EU glyphosate evaluation concluded that the hazard quotient values for intended uses of glyphosate are well below 50, indicating low toxicity for contact and oral routes of exposure according to the EPPO risk assessment scheme. However, the hazard quotient for the highest single application rate of 4320 g a.s./ha was shown to be close to the threshold of 50. It is important to recognize that the hazard quotient for the previous maximum single use rate of 4320 g a.s./ha nearing the threshold of 50 reflects the maximum dose tested being set at the limit dose of 100 µg a.s./bee as well as the endpoint then being >100 µg a.s./bee. Tier 2 summaries have been included for new studies previously not evaluated during the 2001 EU evaluation of glyphosate. These new studies confirm the results of the 2001 evaluation and demonstrate the contact and oral LD_{50} values are >200 µg a.s./bee, providing hazard quotient values well below the threshold of 50 for the proposed maximum single application rate.

IIA 8.7.1 Acute oral toxicity

A summary of the data reviewed in the 2001 EU evaluation of glyphosate acid and new studies not evaluation in the 2001 EU glyphosate evaluation are summarized in Table 8.7.1-1. The data presented below were generated in accordance with OECD or equivalent test guidelines and the appropriate GLP-requirements.

Note this section combines sections 8.7.1 (acute oral toxicity) and 8.7.2 (acute contact toxicity)

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Table 8.7.1-1: Acute toxicity of glyphosate acid and its salts to bees

Test design	Oral LD_{50}	Contact LD_{50}	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate Acid				
48 h [1] oral and contact toxicity	**100 µg a.s./bee**	**>100 µg a.s./bee**	███ 85X094 █████ 1972/no	███ 96-00071
48 h oral and contact toxicity	>200 µg a.s./bee	>200 µg a.s./bee	IIA 8.7.1/01 95 10 48 065 ███ 1995/yes	-
48 h oral and contact toxicity	>40 µg a.s./bee	>20 µg a.s./bee	IIA 8.7.1/02 1413/3-1018 ███, 1996/yes	-
48 h oral and contact toxicity	>182 µg a.s./bee	>103 µg a.s./bee	IIA 8.7.1/03 ███ 9700 ███, 1998/yes	-
72 h oral toxicity	>116.67 µg a.s./bee		IIA 8.7.1/04 141907 ███ 1995/yes	-
72 h contact toxicity	-	>100 µg a.s./bee	IIA 8.7.1/05 142335 ███ 1995/yes	-
Glyphosate-IPA salt				
48 h contact toxicity	-	>61.3 µg a.s./bee >30.44 µg a.e./bee	IIA 8.7.1/06 142335 ███ 2000/yes	-
Glyphosate K-salt				
48 h oral and contact toxicity	>104 µg a.e./bee	>100 µg a.e./bee	IIA 8.7.1/07 ███-2002-108 ███ 2003	-

[1] Study was performed with formulation MON 2139.

Values in bold: Confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

Bee studies on the active substance which were not included in the previous EU-Evaluation of glyphosate (2001) are summarised below.

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Annex point	Author(s)	Year	Study title
IIA 8.7.1/01	[redacted]	1995	Testing Toxicity to Honeybee - *Apis mellifera* L. (laboratory) according to EPPO Guideline No 170. Glyphosate (tec.) [redacted] Report No: 95 10 48 065 Date: 1995-09-11 GLP: yes not published

Guideline:	EPPO Guideline No. 170
Deviations to OECD 213/214:	None
Dates of experimental work:	1995-08-21 to 1995-09-01

Executive Summary

In an acute laboratory study, the oral and contact toxicity of glyphosate acid to the honey bee, *Apis mellifera* L. was tested. Adult worker bees were exposed to two nominal test doses of 100 and 200 µg glyphosate acid/bee.

In the test, three replicate cages, each containing 10 bees were used for the test item treatment, control and reference treatment. Mortality, poisoning symptoms and behavioural abnormalities were recorded 24 and 48 hours after treatment initiation.

Results showed no mortality of bees during the 48 hours test period for test concentrations of up to 200 µg test item/bee (the highest test concentration) in oral and contact toxicity tests. In addition, no behavioural abnormalities were observed in test item groups and control groups during the whole test period. All validity criteria according to the OECD guidelines 213 and 214 were fulfilled.

No effects of glyphosate acid on mortality and behaviour of honey bees were observed at concentrations of up to and including 200 µg glyphosate acid/bee in oral and contact toxicity tests. Therefore, oral and contact LD_{50} of glyphosate acid were determined to be > 200 µg glyphosate acid/bee.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	Not stated
Lot/Batch #:	01/07/95
Purity:	98.2% a.s.
2. Vehicle and/or positive control:	Dimethoate EC 400, containing 411.14 g a.s./L [redacted] (surfactant)
3. Test organisms:	
Species:	Honey bee (*Apis mellifera* L.)
Age:	Adult worker bees
Source:	[redacted] Germany
Diet/Food:	50% aqueous sucrose solution *ad libitum* (except for 1 – 2 hours prior to oral test initiation)
4. Environmental conditions:	
Temperature:	25 - 26°C
Humidity:	53 - 70%
Photoperiod:	8 hours diffuse light / 16 hours darkness

B. STUDY DESIGN AND METHODS

1. Experimental treatments: Oral and contact toxicity tests were conducted with two nominal test doses of 100 and 200 µg glyphosate acid/bee. In addition, for the oral test a control group was fed with 50% sucrose solution. For the contact test, a watery solution of 0.1% [redacted] was used as a negative control. In both tests, dimethoate was used a toxic reference, at test doses ranging from 0.20 to 0.40 µg/bee and 0.0313 to 1.0 µg/bee for oral and contact tests respectively. Both toxicity tests were conducted in triplicate using 10 bees per replicate (30 bees). For oral toxicity test, bees were fed with 50% aqueous sucrose solutions, containing appropriate concentrations of the test item. For contact toxicity test, test solutions containing appropriate concentrations of the test item were dosed to bees by thorax application. After administration of the test substance, the bees were provided with sucrose solution 50%.

2. Observations: Mortality, poisoning symptoms and behavioural abnormalities were recorded 24 and 48 hours after test start.

3. Statistical calculations: Descriptive statistics

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II. RESULTS AND DISCUSSION

A. FINDINGS

The LD_{50}values are given below based on nominal concentrations.

Endpoints (48 h)	Glyphosate acid [µg test item/bee]
Oral LD_{50}	> 200
Contact LD_{50}	> 200

B. OBSERVATIONS

No biologically relevant mortality of bees was observed during the 48-hour test period for test concentrations of up to 200 µg test item/bee, which was the highest concentration tested. In addition, no behavioural abnormalities were observed at any test item concentration and in the control groups.

For the toxic reference dimethoate, the LD_{50} was 0.35 µg test substance/bee for the oral toxicity test and 0.41 µg test substance/bee for the contact toxicity test.

Table 8.7.1-2: Toxicity of glyphosate acid to honey bees in oral and contact toxicity tests

Test	Time [h]	Mortality [%]			
		Control	Glyphosate Acid [µg/bee]		Toxic reference
		-	100	200	Highest test dose*
Oral	24	0	3	0	83
	48	0	3	0	83
Contact	24	0	0	0	97
	48	0	0	0	97

* 0.40 µg/bee for oral toxicity test and 1.0 µg/bee for contact toxicity test

The validity criteria according to the OECD guidelines 213 and 214 were fulfilled as the mortality in the control was < 10% at test termination.

III. CONCLUSION

Under the conditions of the present test, no effects of glyphosate acid on mortality and behaviour of honey bees were observed at concentrations of up to and including 200 µg glyphosate acid/bee in oral and contact toxicity tests. Therefore, both, the oral and contact LD_{50} of glyphosate acid were determined to be > 200 µg glyphosate acid/bee.

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Annex point	Author(s)	Year	Study title
IIA 8.7.1/02	[redacted]	1996	Glyphosate: Acute contact and oral toxicity to honeybees. [redacted] Report No: 1413/3-1018 Date: 1996-08-14 GLP: yes not published

Guideline: EPPO Guideline No. 170: Test methods for evaluating the side-effects of plant protection products on honeybee (1992)

Deviations: none

Dates of experimental work: 1996-06-27 to 1996-07-06

Executive Summary

In an acute laboratory study the oral and contact toxicity of glyphosate acid to honeybee, *Apis mellifera* was tested. After a preliminary dose range-finding test, adult worker bees were treated with 0, 0.625, 1.25, 2.5, 5.0, 10 and 20 µg a.s./bee in the contact test and with 1.25, 2.5, 5.0, 10, 20 and 40 µg a.s./bee in the oral test. Three replicate cages, containing 10 bees each, were used. Mortalities and sub-lethal effects were made 1, 4, 24 and 48 h after treatment. No mortalities or sub-lethal effects were seen in any treatment or controls over the 48 h definitive test period. The validity criteria according to current OECD guidelines 213 and 214 are fulfilled.

In conclusion the 24 and 48-hour oral LD_{50} values for glyphosate acid were > 20 µg a.s./bee for contact exposure (nominal) and > 40 µg a.s./bee for oral exposure (nominal).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: H95 D161A
Purity: 95.3%

2. Vehicle and/or positive control: Reference item: formulated Dimethoate ([redacted])

3. Test organisms:

Species: Honey bee (*Apis mellifera*)
Age: Adult worker bees
Source: [redacted] UK
Diet/Food: 50% sucrose solution *ad libitum*
Acclimatisation: Not stated

4. Environmental conditions:

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Temperature:	24.5 - 25.8°C
Relative humidity:	49.1 - 86.0%
Photoperiod:	darkness

B: STUDY DESIGN AND METHODS

1. Experimental treatments: To determine the test concentrations of glyphosate acid for the definitive study a range-finding test was performed. The nominal doses of glyphosate used for the range-finding test were 0, 0.1, 1, 10 and 20 µg a.s./bee for contact dosing and 0, 0.04, 0.4, 4 and 40 µg a.s./bee for oral dosing.

Contact test: Bees were anaesthetised with carbon dioxide. Contact doses were applied as a 1.0 µL droplet of the test solution was placed on the dorsal thorax of each bee. The nominal doses of glyphosate used for the definitive test contact were 0, 0.625, 1.25, 2.5, 5.0, 10 and 20 µg a.s./bee. The nominal dose of 20 µg a.s./bee was given as a double droplet application (2 x 1 µL). Three replicate cages, containing 10 bees each, were used.

Oral test: The nominal doses of glyphosate used for the definitive oral test were 0, 1.25, 2.5, 5.0, 10, 20 and 40 µg a.s./bee. Three replicate cages, containing 10 bees each, were used. The reference substance was prepared and dosed in the same media and manner as the test substance doses. The toxic standard test was run in concurrently with the range-finding test and shared the controls. The nominal doses of dimethoate were 0, 0.2, 0.4 and 0.8 µg a.s./bee in the contact test and 0, 0.1, 0.15 and 0.2 µg a.s./bee in the oral test. There were three replicate cages of 10 bees each at each dose level of the reference substance.

2. Observations: Assessment of mortalities and sub-lethal effects were made 1, 4, 24 and 48 h after treatment.

3. Statistical calculations: Descriptive Statistics, the LD_{50} values of the toxic standard, dimethoate, were calculated by Probit analysis.

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II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

No mortalities or sub-lethal effects were seen in any treatment or controls over the 48 h definitive test period. The 48 h LD_{50}-values for dimethoate were calculated to be 0.452 µg a.s./bee (95% confidence limits: 0.374 to 0.557) for contact exposure and 0.146 µg a.s./bee (95% confidence limits: 0.131 to 0.161) for oral exposure.

The test is considered to be valid according to OECD guidelines 213 and 214 as mortality in the negative control did not exceed 15% after 48 hours. In addition the LD_{50} for the reference item met the specified range.

III. CONCLUSION

The 24 and 48-hour oral LD_{50} values for glyphosate acid were > 20 µg a.s./bee for contact exposure (nominal) and > 40 µg a.s./bee for oral exposure (nominal).

Annex point	Author(s)	Year	Study title
IIA 8.7.1/03		1998	Glyphosate Acid: Acute Contact and Oral Toxicity to Honey Bees (*Apis mellifera*) Report No: 9700 Date: 1998-12-04 GLP: yes not published

Guideline: EPPO 170 guideline (1992)
OPPTS 850.3020
Draft OECD 213 (1997) and Draft OECD 214 (1997)

Deviations: The starvation of bees before test initiation was 2 h and 10 min, instead of 1-2 h. This is not supposed to have an effect on the reliability of the study.

Dates of experimental work: 1998-08-24 to 1998-09-04

Executive Summary

In an acute laboratory study the contact and oral toxicity of glyphosate acid to the honey bee, *Apis mellifera* L., were tested. Following a range finding test, a definitive test was conducted exposing female worker bees to nominal doses of 0.0984, 0.984, 9.84 and 103 µg test item/bee. In the oral toxicity test, 206 µg a.s./bee was tested additionally.

In both test setups, three replicate cages, each containing 10 bees, were used for the test item treatments, controls and reference treatments. Mortality and sub-lethal effects were assessed 4, 24 and 48 h after test initiation for contact and oral toxicity.

No mortality of bees was observed after 72 hours of exposure. In addition, no sub-lethal effects were observed in the test item and the control groups during the 72 hours test period. All validity criteria according to OECD 213 and OECD 214 were fulfilled.

In conclusion, the toxicity of glyphosate acid was tested in an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >103 µg a.s./bee in the contact toxicity test, in the oral toxicity test it was > 182 µg a.s./bee.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Description:	White powder
Lot/Batch #:	TSC 0521/05148
Purity:	97.6%

2. Vehicle and/or positive control:
Vehicle for test item: Agral 90
Vehicle for positive control: Triton X100
Positive control: Dimethoate ( )

3. Test organisms:

Species: Honey bee (*Apis mellifera* L.)
Age: Adult worker bees
Source: [redacted]
Diet/Food: Not stated

4. Environmental conditions:

Temperature: 25 ± 1°C
Humidity: 65 ± 5%
Photoperiod: 24 hours darkness (except during observation)

B: STUDY DESIGN AND METHODS

1. Experimental treatments:

Contact test: The definitive test was conducted with 0.0984, 0.984, 9.84 and 103 µg glyphosate acid/bee prepared in an appropriate carrier (deionised water containing 500 mg/L of the wetting agent Agral 90) and administered as a 1.0 µL droplet per bee (dorsal thorax) to each of ten bees in each of three cages per treatment. A control with 500 mg Agral 90/L and a toxic reference solution containing 1g Triton X100/L were run in parallel. During the observation method a 50% w/v aqueous sucrose solution was provided.

Oral test: The definitive test was conducted with 0.0984, 0.984, 9.84, 103 and 206 µg glyphosate acid/bee, in 50% w/v aqueous sucrose solution, the formulation having first been dissolved in deionised water containing 500 mg/L Agral 90. A control of 0.5 mL water and the toxic reference solution containing 1 g Triton X100/L were prepared analogically.

The treated food was offered in glass test feeders, which were weighed before and after introduction into the three cages per treatment. Each replicate cage contained ten bees. Duration of uptake was 4 hours for the test item treatments. At the highest treatment level the mean dose consumed was 182 µg glyphosate acid/bee.

2. Observations: Mortality and sub-lethal effects were assessed 4, 24 and 48 h after test initiation for contact and oral toxicity.

3. Statistical calculations: Doses and LD_{50} calculations were based on the analysed content of glyphosate acid. The mortality results were analysed using by Probit analysis.

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II. RESULTS AND DISCUSSION

A. FINDINGS

Table 8.7.1-3: Toxicity of glyphosate acid to honey bees (*Apis mellifera*) in the contact and oral toxicity test

Dose [µg a.s./bee]	Mean intake of test item [µg a.s./bee]	Mortality [%]		
		24 h	48 h	72 h
Contact toxicity test				
Control	-	0	0	0
0.0984	-	0	0	0
0.984	-	0	0	0
9.84	-	0	0	0
103	-	0	0	0
Oral toxicity test				
Control	-	0	0	0
0.0984	0.0947	0	0	0
0.984	0.937	0	0	0
9.84	9.7	0	0	0
103	80	0	0	0
206	182	0	0	0

B. OBSERVATIONS

In both test setups, no mortality of bees was observed in the 72 hours test period. In the oral toxicity test the maximum nominal test level of 206 µg a.s./bee) corresponded to an actual intake of 182 µg a.s./bee. In addition, no sub-lethal effects were observed in the test item group and the control group during the 72 hours test period.

All validity criteria according to OECD 213 and OECD 214 were fulfilled, since the average mortality in the control group did not exceed 10% and the LD_{50} of the toxic standard meets the specified range.

III. CONCLUSION

The toxicity of glyphosate acid was tested in an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >103 µg a.s./bee in the contact toxicity test, in the oral toxicity test it was > 182 µg a.s./bee.

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Annex point	Author(s)	Year	Study title
IIA 8.7.1/04		1995	Honey Bees (*Apis mellifera L.*), oral toxicity study in the laboratory with Glyphosate Report No: 141907 Date: 1995-09-22 GLP: yes not published

Guideline: EPPO 22, 203-215 (1992)

Deviations to OECD 213: None

Dates of experimental work: 1995-03-08 to 1995-03-16

Executive Summary

In an acute laboratory study the oral toxicity of glyphosate acid to the honey bee, *Apis mellifera* L., was tested. Following a range finding test, a definitive test was conducted exposing worker bees to a single nominal dose of 121 µg test item/bee.

In the test, three replicate cages, each containing 10 bees, were used for the test item treatments, controls and reference treatments. Paralysis and mortality effects were recorded at least the following approximate time intervals: 30, 60, 90 and 120 minutes after treatment and 24, 48and 72 hours after treatment.

No mortality of bees was observed after 72 hours of exposure. In addition, no paralysis was observed in the test item and the control groups during the 72 hours test period. The validity criteria according to guideline OECD 213 are fulfilled.

In an oral toxicity test, glyphosate acid had no effects on mortality of honey bees at concentrations of up to and including 116.67 µg a.s./bee. Therefore, the oral LD_{50} of glyphosate acid was determined to be >116.67 µg a.s./bee.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: 22021
Purity: 96%

2. Vehicle and/or positive control: Parathion 25% liquid

3. Test organisms:

Species: Honeybee (*Apis mellifera* L.)
Age: Adult worker bees
Source:
Diet/Food: 50% aqueous sucrose solution *ad libitum* (except during oral dosing and prior starvation)

4. Environmental conditions:

Temperature: 24- 25°C
Humidity: 34 - 37%
Photoperiod: 24 hours darkness (except during observation)

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Prior to the main test, a range-finding test was performed exposing bees to nominal glyphosate acid concentrations of 1.0, 10, 51 and 101 µg a.s./10 µL sucrose solution. The definitive test was conducted as a limit test with a single nominal concentration of 121 µg test item/10 µL sucrose solution. All test solutions were prepared in a 50% sucrose solution. In addition a water-treated control and a reference substance (Parathion 25% liquid) were tested.

For the test, 10 bees per cage were exposed in triplicate and fed with the test substance suspension. Per group of 10 bees 100 µL test substance suspension was administered (10 µL test solution/bee).

2. Observations: Mortality, paralysis and any other abnormalities were recorded at least the following approximate time intervals: 30, 60, 90 and 120 minutes after treatment and 24, 48and 72 hours after treatment start.

3. Statistical calculations: Descriptive statistics.

II. RESULTS AND DISCUSSION

A. FINDINGS

Table 8.7.1-4: Toxicity of glyphosate acid to honey bees (*Apis mellifera*) in an oral toxicity test

Dose [µg test item/bee]	Intake of test item [µg a.s./bee]	Mortality [%]		
		24 h*	48 h*	72 h*
Control (sugar solution)	-	0.00	3.33	3.33
Definitive test: glyphosate				
121	116.67	0.00	0.00	0.00

* Corrected for mortality in the negative control

B. OBSERVATIONS

Oral toxicity test: No mortality of bees was observed at the in the 72 hours test period for the test concentration of 121 µg test item/bee, which is the highest test concentration. In addition, no paralysis was observed in the test item group and the control group during the 72 hours test period.

The test is considered to be valid according to OECD guideline 213 as mortality in the negative control did not exceed 15% after 48 hours. In addition the LD_{50} for the reference item met the specified range.

III. CONCLUSION

In an oral toxicity test, glyphosate acid had no effects on mortality of honey bees at concentrations of up to and including 116.67 µg a.s./bee. Therefore, the oral LD_{50} of glyphosate was determined to be >116.67 µg a.s./bee.

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Annex point	Author(s)	Year	Study title
IIA 8.7.1/05		1995	Honey Bees (*Apis mellifera L.*), contact toxicity study in the laboratory with Glyfosaat Report No: 142335 Date: 1995-09-22 GLP: yes not published

Guideline: EPPO 22, 203-215 (1992)

Deviations to OECD 214: None

Dates of experimental work: 1995-03-20 to 1995-03-25

Executive Summary

In an acute laboratory study the contact toxicity of glyphosate acid to the honey bee, *Apis mellifera* L. was tested. Following a range finding test, adult worker bees were exposed to a single nominal dose of 100 µg test item/bee.

In the test, three replicate cages, each containing 10 bees, were used for the test item treatment, control and reference treatment. Mortality and paralysis effects were recorded at least at the following approximate time intervals: 30, 60, 90 and 120 minutes after treatment and 24, 48 and 72 hours after treatment.

No mortality of bees was observed during the 72 hours of exposure. In addition, no paralysis was observed in the test item groups and the control groups during the 72 hours test period. The validity criteria according to guideline OECD 214 are fulfilled.

In a contact toxicity test, no effects of glyphosate acid on the mortality and the paralysis of honey bees were observed at concentrations up to and including 100 µ g a.s./bee. The contact LD_{50} of glyphosate was therefore determined to be>100 µ g a.s./bee.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: 22021
Purity: 96%

2. Vehicle and/or positive control: Acetone (vehicle), Parathion 25% liquid (positive control)

3. Test organisms:

Species: Honey bee (*Apis mellifera* L.)
Age: Adult worker bees

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Source: [redacted]

Diet/Food: 50% aqueous sucrose solution *ad libitum* (except during the treatment)

4. Environmental conditions:

Temperature: 24 – 25°C

Humidity: 34 – 40%

Photoperiod: 24 hours darkness (except during observation)

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Prior to the main test, a range-finding test was a.s./item/bee. The definitive test was conducted as a limit test with a single nominal dose of 100 µg test item/bee. In addition, a negative control constituted of acetone and the reference substance Parathion 25% liquid were tested.

For the definite test, adult worker bees were exposed in triplicates (10 bees/test cage) to the test item, control and reference item. After administration of the test substance, the bees were provided with sucrose solution 50%.

2. Observations: Mortality, paralysis and any other abnormalities were recorded at least the following approximate time intervals: 30, 60, 90 and 120 minutes after treatment and 24, 48 and 72 hours after treatment start.

3. Statistical calculations: Descriptive statistics.

II. RESULTS AND DISCUSSION

A. FINDINGS

Table 8.7.1-5: Toxicity of glyphosate acid to honey bees (*Apis mellifera*) in a contact toxicity test

Dose [µg a.s./bee]	Mortality [%]		
	24 h	48 h	72 h
Control (Acetone)	0.00	0.00	0.00
100	0.00	0.00	0.00

B. OBSERVATIONS

Contact toxicity test: No mortality of bees was observed during the 72 hours test period for the test concentration of 100 µg test item/bee. In addition, no paralysis was observed in test item groups and control groups during the 72 hours test period.

Validity: The test is considered to be valid according to OECD 214 as mortality in the negative control did not exceed 15% and the LD_{50} of the toxic standard met the range specified.

III. CONCLUSION

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In a contact toxicity test, no effects of glyphosate acid on mortality of honey bees were observed at concentrations up to and including 100 µg a.s./bee. The contact LD_{50} of glyphosate was therefore determined to be>100 µg a.s./bee.

Annex point	Author(s)	Year	Study title
IIA 8.7.1/06	█	2000	Acute Contact Toxicity of GLIFOSATO IPA TECHNICO NUFARM to Honey Bees (*Apis mellifera L.*) █ Report No: █-D4.017/00 Date: 2000-07-24 GLP: yes not published

Guideline: OECD Draft Proposal for a New Guideline: Honey bees, Acute Contact Toxicity Test (1996).

Deviations: None

Dates of experimental work: 2000-06-05 to 2000-06-14

Executive Summary

In an acute laboratory study the contact toxicity of the isopropylamine (IPA) salt of glyphosate to the honey bee, *Apis mellifera* was tested. Following a range finding test, adult worker bees were exposed to nominal dose rates of 10.0, 12.5, 24.0, 62.5 and 100.0 µg test item/bee. In addition, an undosed control was tested. Technical dimethoate was used as a reference item.

In the test, three replicate cages, each containing 10 bees, were used for the test item treatment, control and reference treatment. Mortality and sublethal effects were recorded at 24 and 48 hours after the treatment.

No significant mortality of bees was observed during the 48 hours observation period. In addition, no sublethal effects were observed. The validity criteria according to guideline OECD 214 are fulfilled.

In conclusion, under the conditions of the present test, the 48 h contact LD_{50} of glyphosate IPA salt was determined to be >100 µg glyphosate IPA salt/bee, equivalent to >30.44 µg a.e./bee.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine salt (technical)
Description:	Not stated
Lot/Batch #:	MJRT 02 S 201 04
Purity:	612.7 g/kg salt equivalent (analysed), equivalent to 304.4 g a.e./kg
Density:	Not stated

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2. Vehicle and/or positive control: Reference item: technical Dimethoate

3. Test organisms:

Species: Honey bee (*Apis mellifera*)
Age: Adult worker bees from healthy colonies
Source: [redacted] Brasil
Diet/Food: Sucrose solution *ad libitum*
Acclimatisation: At 25 ± 2°C and 65 ± 5 % relative humidity between collection of worker bees and test initiation (time span not stated)

4. Environmental conditions:

Temperature: 27 – 31°C
Relative humidity: 40 – 67 %
Photoperiod: 24 hours darkness

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Based on the results of a range-finder test, bees in the main test were exposed to the nominal dose rates of 10.0, 12.5, 24.0, 62.5 and 100.0 µg test item/bee. In addition, an undosed control was tested. Technical dimethoate was used as a reference item. The test was conducted with 3 replicates (cages) per test concentration/control and 10 bees per cage. Bees were exposed to the test item by administering 1.0 µL of the test substance or reference item on the ventral surface of the thorax with a micro syringe. After dosing the cages were kept in darkness for 48 hours. Sucrose solution was available *ad libitum* throughout the whole test period.

2. Observations: Mortality and sublethal effects were recorded at 24 and 48 hours after treatment.

3. Statistical calculations: Descriptive statistics for the test item. Data on mortality for dimethoate were analysed using trimmed Spearman-Karber Method.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Analytical determination of the test concentrations showed that the deviation from the nominal concentrations was not higher than 20 %. Therefore, the ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

Table 8.7.1-6: Toxicity of glyphosate isopropylamine salt to honey bees (*Apis mellifera*) in a contact toxicity test

Dose [µg test item/bee]	Mortality (mean of 3 replicates) [%]	
	24 h	48 h
Control (undosed)	0.0	0.0
10.0	0.0	0.0
12.5	0.0	0.0
24.0	0.0	0.0
62.5	0.0	0.0
100.0	3.33	3.33

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Reference test: The determined 48h LD_{50} for the reference item dimethoate was 0.12 mg/L. These results show a toxicity level within the ranges reported by the OECD guidelines showing that bees were reacting normally in this test.

B. OBSERVATIONS

No sublethal effects were observed up to a dose of 100 µg test item/bee, equivalent to 30.44 µg a.e./bee. The test is considered to be valid according to OECD 214 as mortality in the negative control did not exceed 15% and the LD_{50} of the toxic standard met the range specified.

III. CONCLUSION

Under the conditions of the present test, the 48 h contact LD_{50} of glyphosate IPA salt was determined to be >100 µg glyphosate IPA salt/bee, equivalent to >30.44 µg a.e./bee.

Annex point	Author(s)	Year	Study title
IIA 8.7.1/07	[redacted]	2003	Laboratory bioassays to determine acute oral and contact toxicity of MON 78623 to the honeybee, *Apis mellifera* [redacted] [redacted] Report No: [redacted]-2002-108 Date: 2003-04-11 GLP: yes not published

Guideline: EPPO guideline 170 (1992)

Deviations from OECD 213 & 214: -

Dates of experimental work: Not stated

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Executive Summary

In an acute laboratory study the contact and oral toxicity of glyphosate K-salt to the honey bee, *Apis mellifera* L., were tested. Following a range finding test, a definitive test was conducted exposing worker bees to nominal doses of 100 µg glyphosate acid equivalent/bee.

In both test setups, five replicate cages, each containing 10 bees, were used for the test item treatments, controls and three for the reference treatments. Mortality and sub-lethal effects were assessed 1, 3, 24 and 48 h after test initiation for contact and oral toxicity.

Corrected mortality for contact toxicity was 0% and for oral toxicity 4%. No sublethal effects were observed except for one bee one hour after test item application. All validity criteria according to OECD 213 and OECD 214 were fulfilled.

In conclusion, the toxicity of glyphosate K-salt was tested in an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >100 µg glyphosate acid equivalent/bee in the contact toxicity test, in the oral toxicity test it was > 104 µg glyphosate acid equivalent/bee.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 78623
Description: Amber liquid
Lot/Batch #: GLP-0108-11688-F
Purity: 47.3%

2. Vehicle and/or positive control: Vehicle for test item: wetting agent Farmon Blue (87.3% w/w alkyl phenol ethylene oxide)
Positive control: Dimethoate technical grade

3. Test organisms:

Species: Honey bee (*Apis mellifera* L.)
Age: Adult worker bees
Source:
Diet/Food: 50% w/v aqueous sucrose solution

4. Environmental conditions:

Temperature: 25 – 26°C
Humidity: 64 – 79%
Photoperiod: 24 hours darkness (except during observation)

B: STUDY DESIGN AND METHODS

1. Experimental treatments:

Contact test: Following an initial range-finding test, the definitive test was conducted as a limit test with 100 µg glyphosate acid equivalent/bee, prepared in an appropriate carrier (0.05% solution of the wetting agent Farmon Blue)and administered as a 1.0 µL droplet per bee (dorsal thorax) to each of ten bees in each of five cages per treatment. A vehicle control containing 0.05 w/v solution of Farmon Blue and deionised water and a toxic reference solution containing dimethoate were run in parallel. During the observation method a 50% w/v aqueous sucrose solution was provided.

Oral test: The definitive test was conducted with 100 µg test item/bee, in 50% w/v aqueous sucrose solution. A control of deionised water and the toxic reference solution containing dimethoate were prepared analogically.

The treated food was offered in narrow glass vials, which were weighed before and after introduction into the three cages per treatment. Each replicate cage contained ten bees. At the highest treatment level the mean dose consumed was 104 µg a.e./bee.

2. Observations: Mortality and sub-lethal effects were assessed 1, 3, 24 and 48 h after test initiation for contact and oral toxicity.

3. Statistical calculations: Corrected mortality was calculated according to Abbott (1925). LC_{50} values were determined by Probit analysis and the 95% confidence interval by Chi-square goodness of fit test.

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II. RESULTS AND DISCUSSION

A. FINDINGS

Table 8.7.1-7: Toxicity of glyphosate K-salt to honey bees (*Apis mellifera*) in the contact and oral toxicity test

Dose [µg a.e./bee]	Mean intake of test item [µg a.e./bee]	Mortality [%]			
		1	3	24 h	48 h
Contact toxicity test					
Control	-	0	0	2	4
Farmon Blue control	-	0	0	2	4
100	-	0	0	2	2 (0)
Oral toxicity test					
Control	-				6
100	104				10 (4)

In brackets the Abbot corrected mortality is given

B. OBSERVATIONS

<u>Contact toxicity</u>: Corrected mortality at 48 h was 0%. No sublethal effects were observed except for one bee one hour after test start, but it recovered by 3 h.

<u>Oral toxicity test</u>: No sublethal effects of bees were observed during the 48 hour test period for the test concentration of 104 µg glyphosate acid equivalent/bee and the sucrose control. The corrected mortality after 48 h was 4%.

<u>Reference test:</u> The determined contact 48h LD_{50} for the reference item dimethoate was 0.123 µg/bee and 0.126 µg/bee for oral toxicity. These results are in line with published values, indicating that the test insects were of suitable sensitivity.

The test is considered to be valid according to OECD guideline 213 as mortality in the negative control did not exceed 15% after 48 hours. In addition the LD_{50} for the reference item met the specified range

All validity criteria according to OECD 213 and OECD 214 were fulfilled, since the average mortality in the control group did not exceed 10% and the LD_{50} of the toxic standard meets the specified range.

III. CONCLUSION

The toxicity of glyphosate acid was tested in an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >100 µg glyphosate acid equivalent/bee in the contact toxicity test, in the oral toxicity test it was > 104 µg glyphosate acid equivalent/bee.

IIA 8.7.2 Acute contact toxicity

Please refer to IIA 8.7.1 and studies IIA 8.7.1/01 – II 8.7.1/07 for a review of acute contact toxicity, which presents a combined summary of acute contact and oral toxicity.

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IIA 8.7.3 Exposure of residues on foliage to honey bees

Below is a summary of an exposure study performed in support of the experimental design and dose setting the bee brood study and does not evaluate the toxicity to bees.

Annex point	Author(s)	Year	Study title
IIA 8.7.3/01	[redacted]	2011	Glyphosate: Study to determine potential exposure of honeybee colonies to residues under semi-field conditions [redacted] Report No: [redacted] 1002 Date: 2011-12-21 GLP: yes not published

Guideline: None; tailor made study

Deviations: None

Dates of experimental work: 2011-05-20 to 2011-05-27

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Executive Summary

A semi-field study was undertaken to determine the potential exposure of honeybee colonies to glyphosate by quantifying residues in relevant food matrices, i.e. pollen and nectar, when the formulation MON 52276 was applied to flowering *Phacelia* grown in two large (180 m²) glasshouses. Following treatment of nominal 8 L/ha, equivalent to 2.88 kg a.e./ha, two honey bee colonies per glasshouse were exposed. Foraging activity in the crop and activity at each hive was assessed daily for 7 days. On days 0, 1, 2, 3, 4 and 7, forager bees were taken to get hold of the nectar from the honey stomach of the bees after foraging in the treated crop. On days -1, 1, 2, 3, 4 and 7, samples of pollen were collected from the pollen traps fitted to each hive. Samples of nectar were also collected from the combs in each hive on day 7. Furthermore, samples of larvae were collected from the combs in each hive on days 4 and 7. Daily assessments were made of the percentage of plants with wilted leaves or flowers.

Foraging assessment showed foraging activity on the crop from start of study throughout the exposure period in glasshouse 1 with a peak on day 4. The lowest foraging activity was observed on day 5 at 38% of the mean pre-spray activity. In glasshouse 2 the activity declined throughout the assessment period to reach less than 10% of mean spray activity on days 5-7. In line with the decreased foraging activity in glasshouse 2, the crop started to show significant effects of the treatment from day 4 onwards.

Residues in nectar samples taken from forager bees at various time points after application ranged from 2.78 to 31.3 mg a.e./kg; residues in nectar samples taken from the colonies ranged from below LOQ (1.0 mg a.e./kg) to 1.30 mg a.e./kg. Residues in pollen samples taken from the pollen trap at various time points after application ranged from 87.2 to 629 mg a.e./kg. Residues in larvae samples ranged from 1.23 to 19.50 mg a.e./kg.

The residue data can be used to assess the approximate exposure level of brood within colonies exposed under worst-case conditions.

The maximum pollen collected per colony was 2.9 g on day 0 and the traps are estimated to be about 50% efficient so about 6 g of pollen per day was returned to the hive (the colony is using about 4.5 g of this based on the [redacted] 2005).

The nectar can be assessed using a mean of 18 foragers returning to the hive per 30 seconds and approximately 50 µL per load (max), which gives 18 trips/30 sec * 60 sec/min * 60 min/hour * 12 hours

max foraging/day, equal to 25,920 trips/day * 0.050 mL, resulting in 1296 mL/day (of which the colony is using 135 g based on [redacted] 2005).

As a worst case example considering the colony size of the present study, a honey bee colony collects 6 g pollen and 1296 mL nectar and of this the brood consumes 4.5 g pollen and 135 g nectar, which allows the excess to be stored for later consumption. As simulated in this study, for honeybee colonies foraging on the model crop *Phacelia* treated with 8 L MON 52276/ha, a total daily intake of glyphosate residues of 44.0 mg a.e. (based on day 1 maximum mean residues) and of 22 mg a.e. (based on mean residues over days 1-3) can be estimated.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 52276 (Soluble concentrate)
Active substance:	Glyphosate acid 360 g glyphosate acid equivalents/L (nominal)
Active substance content:	358.8 g glyphosate acid equivalents/L (according to the Certificate of Analysis)
Proposed use:	Herbicide
Description:	Clear brown liquid
Lot/Batch #:	A9K0106104
Density:	1.1693 g/mL at 20°C (according to the Certificate of Analysis)

2. Vehicle and/or positive control: None

3. Test organism:

Species:	*Apis mellifera* 4 honey bee colonies containing 4 – 6 frames of brood, containing 6000 – 12000 adult bees
Age:	Not stated
Source:	UK [redacted]
Acclimatisation:	3 days

4. Test system: Two 180 m² glasshouses at [redacted] U.K.

Crop cultivated:	*Phacelia* (sown directly into soil of the glasshouse, no pesticide use during cultivation)
Replication:	2 glasshouses, each containing 2 bee colonies

5. Environmental conditions:

Temperature:	Glasshouse 1: 7.7 – 39.9 °C, temperatures of >35 °C were recorded on day 6 and 7 for 10 and 30 min. Glasshouse 2: 8.3– 47.4 °C, temperatures of >35 °C were recorded on days -1, 1, 2, 4, 6 and 7 for up to 30 min until day 4, for 1.5 h on day 4, 50 min on day 6 and 40 min on day 7. High temperatures occurred primarily between 11:30 and 14:00 and exhibited no obvious effects on crop or foraging bees
Humidity:	Glasshouse 1:

19.5 to 93.4%

Glasshouse 2:
13.9 to 100%

B: STUDY DESIGN AND METHODS

1. Experimental treatments:

Study site: The study was conducted in two 180 m² glasshouses situated at [redacted] [redacted]. The glasshouses were well ventilated (but equipped with insect proof) to be as representative as possible of the outdoor situation but without direct precipitation. *Phacelia* was planted directly into the soil inside the glasshouse and no pesticides were applied during cultivation. The timing of the start of test i.e. transfer of colonies into the glasshouse was determined by the flowering of the crops. Temperature and humidity in the glasshouses was recorded continuously.

Experimental design: Four colonies of bees and brood comprising each of 4 to 6 frames of brood and containing 6000 to 12000 adult bees were used. Hives were fitted with a pollen trap. Three days prior to application two colonies each were located on opposite sides of each glasshouse and allowed to fly freely within the glasshouse. Colonies A and B were placed in glasshouse 1, colonies C and D were placed in glasshouse 2.

Test item application: The test item MON 52276 (nominal content: 360 g glyphosate acid equivalent/L) was applied onto the crop grown in the glasshouse on day 0 during a period when bees were actively foraging using a 3 nozzle lunch box sprayer unit with a hand-held boom fitted with Lurmark 03 F110 nozzles. The sprayer was pre-calibrated to deliver a known application rate of 400 L/ha. The colonies were protected from direct overspray and spray drift during the application.

2. Observations: Foraging assessments were performed each day during times peak foraging activity. The assessments were performed by counting the number of bees foraging in a marked area (5 times in 1 m transects) during a 1 minute period during peak activity. In addition, the number of bees returning to each hive and the number carrying pollen loads were counted during a 30 second period.

Visual assessment of the crop was performed daily by determination of the proportion of plants with wilted flowers and wilted leaves.

The contents of the pollen traps were collected on days -1, 1, 2, 3, 4 and 7 after application. Samples of forager bees were collected on days 0, 1, 2, 3, 4 and 7 after application. The nectar was collected from the bees honey stomachs. On days 4 and 7 samples of ten 4-5 day old larvae were taken from each colony, on day 7 an additional sample of nectar was collected from the combs of each colony.

3. Residues analysis: Analysis of glyphosate acid in samples was conducted following extraction with acetonitrile:water (1:4, v/v), clean up by solid phase extraction on C18 and derivatisation as FMOC-glyphosate and a second clean up (solid phase extraction on Oasis HLB, methanolic elution) by HPLC-MS/MS. Limit of quantification (LoQ) and limit of detection (LoD) were 1.0 and 0.3 mg/kg, respectively.

4. Data analysis: Considering residue levels determined in nectar and pollen after treatment of a model crop, possible exposure scenarios of honeybee brood are estimated based on information available from literature and the present study.

II. RESULTS AND DISCUSSION

A. FINDINGS

Verification of test item application: The actual application rates were 8.19 L MON 52276/ha (2.94 kg a.e./ha) in glasshouse 1 and , 8.30 L MON 52276/ha (2.98 kg a.e./ha) in glasshouse 2. The application rate

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was 102 – 104% of the nominal application rate of 8 L MON 52276/ha and 102-103% of the nominal application rate of 2.88 kg a.e./ha.

Residue analysis: Residues in nectar samples taken from forager bees at various time points after application ranged from 2.78 to 31.3 mg a.e./kg; residues in nectar samples taken from the colonies ranged from below LOQ (1.0 mg a.e./kg) to 1.30 mg a.e./kg. Residues in pollen samples taken from the pollen trap various times after application ranged from 87.2 to 629 mg a.e./kg. Residues in larvae samples ranged from 1.23 to 19.50 mg a.e./kg.

Table 8.7.3-1: Summary of residue analysis of pollen, nectar and larvae samples.

		Days after treatment [mg glyphosate acid equivalent/kg]					
	Hive	**-1**	**1**	**2**	**3**	**4**	**7**
Nectar (honey stomachs)	A+B	n.d.	25.5	9.24	4.90 (samples combined DAT 3, 4, 7)		
	C+D	n.d.	31.3	15.2	7.18 (samples combined DAT 3, 4)		2.78
	Overall mean	**n.d.**	**28.4**	**12.2**	**6.0**		
Nectar (hive)	A	-	-	-	-	-	<LoQ
	B	-	-	-	-	-	1.30
	C	-	-	-	-	-	1.06
	D	-	-	-	-	-	1.00
Mean							**0.99**
Larvae (comb)	A	-	-	-	-	8.32	2.54
	B	-	-	-	-	16.70	10.6
	C	-	-	-	-	19.50	6.72
	D	-	-	-	-	2.88	1.23
Mean						**11.9**	**5.3**
Pollen (pollen trap)	A	n.d.	325	255	119 (samples combined)	134 (samples combined)	87.2 (samples combined)
	B	n.d.	405	213			
	Mean A&B	-	**365**	**234**	**119**	**134**	**87.2**
	C	n.d.	518	333	181	176	130 (samples combined)
	D	n.d.	629	477	147	180	
	Mean C&D	-	**573.5**	**405**	**164**	**178**	**130**
	Overall mean	-	**469**	**320**	**142**	**156**	**109**

DAT day after treatment
n.d. not detected
LOQ 0.6 mg/kg
LOD 0.3 mg/kg

B. OBSERVATIONS

Foraging activity: Foraging assessment showed foraging activity on the crop from start of study throughout the exposure period in glasshouse 1 with a peak on day 4. The lowest foraging activity was observed on day 5 at 38% of the mean pre-spray activity. In glasshouse 2 the activity declined throughout the assessment period to reach less than 10% of mean spray activity on days 5-7. In line with the decreased foraging activity in glasshouse 2, the crop started to show significant effects of the treatment from day 4 onwards.

Data analysis: The residue data can be used to assess the approximate exposure level of brood within colonies exposed under worst-case conditions.

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Table 8.7.3-2: Assessment of possible exposure of honey bee colonies to glyphosate residues under two scenarios is depicted below.

Scenario	Daily intake of glyphosate residues in nectar (1296 g nectar/d) [mg]	Daily intake of glyphosate residues in pollen (6 g pollen/d) [mg]	Total daily intake of glyphosate residues [mg a.e.]
Day 1 maximum mean residues (31.3 µg a.e./g in nectar, 574 µg a.e./g in pollen, glasshouse 2)	40.6	3.4	44.0
Mean residues over days 1-3 (15.5 µg a.e./g in nectar, 310 µg a.e./g in pollen, both glasshouses)	20.1	1.9	22.0

Two approaches can be made to assessing exposure - one based on generic published data on the requirements for nectar and pollen by larvae (generic data) and the other based on the observations made in this study (study data).

Generic data: The calculations are based on a daily brood requirement of 30 mg nectar (based on 40% sugar in nectar) and 1 mg pollen for worker brood (2005). Based on a brood frame being 3600 cells and 25% of the time is as unsealed brood (hatch day 3 to sealed day 8 with emergence day 21) then five frames of brood (4-6 were used in this study) is 18,000 brood cells therefore for 4500 larvae with a requirement of 135 g/day nectar and 4.5 g/day pollen for the colony.

Study data: The second approach is to assess the amount of pollen and nectar returning to the hive over the time course of exposure using the data on the numbers of returning foragers in the study and the amounts of pollen and nectar collected from bees by using the pollen trap and individual bee samples.

The maximum pollen collected per colony was 2.9 g on day 0 and the traps are estimated to be about 50% efficient so about 6 g of pollen per day was returned to the hive (the colony is using about 4.5 g of this based on the 2005).

The nectar can be assessed using a mean of 18 foragers returning to the hive per 30 seconds and approximately 50 µL per load (max), which gives 18 trips/30 sec * 60 sec/min * 60 min/hour * 12 hours max foraging/day, equal to 25,920 trips/day * 0.050 mL, resulting in 1296 mL/day (of which the colony is using 135 g based on 2005).

III. CONCLUSION

As a worst case example considering the colony size of the present study, a honey bee colony collects 6 g pollen and 1296 mL nectar and of this the brood consumes 4.5 g pollen and 135 g nectar, which allows the excess to be stored for later consumption. As simulated in this study, for honeybee colonies foraging on the model crop *Phacelia* treated with 8 L MON 52276/ha, a total daily intake of glyphosate residues of 44.0 mg a.e. (based on day 1 maximum mean residues) and of 22 mg a.e. (based on mean residues over days 1-3) can be estimated.

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IIA 8.7.4 Bee brood feeding test

Annex point	Author(s)	Year	Study title
IIA 8.7.4/01	[redacted]	2012	Glyphosate: Evaluating potential effects on honeybee brood (*Apis mellifera*) development [redacted] Report No: [redacted]1001 Date: 2012-01-27 GLP: yes not published

Guideline: Oomen *et al.*, 1992

Deviations: Some colonies used in the study were slightly smaller in terms of the number of brood frames, but this was not considered to have a significant impact on the study as all were viable colonies at the start of the study and a sufficient number of brood cells was available for detailed observations.
Feeding period was extended up to 5 days. This extension of the feeding period is not considered to have had an impact on the validity of the study.

Dates of experimental work: 2011-06-21 to 2011-08-23

Executive Summary

A field study was undertaken to determine the potential for toxicity to developing honey bee larvae and pupae to glyphosate (tested as the IPA salt) when fed directly to honey bee colonies. The IPA salt was selected as the test substance because it is representative of the active substance in glyphosate formulations and the appropriate for this terrestrial study. Three groups of four colonies were treated with 75, 150 and 301 mg a.e./L of glyphosate in 1 litre of 50% w/v sucrose. One group of four colonies was fed with 1 litre 50% w/v sucrose solution only and one group of four colonies was fed with the toxic reference fenoxycarb dispersed in 1 litre of 50% w/v sucrose. Brood cells were marked in each colony (100 cells containing eggs, 100 cells containing 1-2 day old larvae, and 100 cells containing 3-4 day old larvae) up to 24 hours to dosing using the standard acetate overlay method. On day 7 and just prior to expected emergence, the marked brood cells (eggs, young and old larvae) were assessed for mortality and appearance in each test colony. The content of the dead bee traps attached to the colonies was counted daily during brood assessment period. All colonies were assessed within one week prior to the dosing and within week 1, 2 and 3 after dosing. Samples of each concentration of test item treated sucrose solution were taken on the day of dosing. Four to five day old larvae were sampled 4 and 7 days following start of dosing.

Measured glyphosate (a.e.) concentrations in the dosing solutions were within 11% of the nominal doses. Mean measured glyphosate (a.e.) residues in larvae on 4 days were 13, 37 and 53 mg a.e./kg for the nominal dose levels of 75, 150, and 301 mg a.e./L. Mean measured residues after 7 days were reduced with values of 1.7, 3.2 and 4.1 mg a.e./kg for the nominal dose levels of 75, 150, and 301 mg a.e./L. Glyphosate acid was not detected in the control group.

No biologically significant adult mortality was observed in any treatment group. Over a 16 day observation period after dosing, 2.0 dead pupae/colony were observed in the control and 1.3 – 1.8 dead pupae/colony were observed in the glyphosate treated colonies. Overall survival was 85% for marked eggs, 96% for marked young larvae and 96% for marked old larvae in controls and 82-87% for marked eggs, 87-94% for marked young larvae and 94-95% for marked old larvae in the glyphosate treated colonies.

The overall NOAEL for brood development of honey bees was the highest dose tested – 301 mg glyphosate acid equivalent/L (nominal) equivalent to 245 mg glyphosate acid equivalent/kg nominal when considering the density of the sucrose solution and 266 mg glyphosate acid equivalent/kg actually measured.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 0139

Active substance: Glyphosate isopropylamine salt

Active substance content: 62.27% Glyphosate isopropylamine salt
46.14% glyphosate acid equivalents/L (measured)

Description: Clear light yellow liquid

Lot/Batch #: GLP-1104-21370-T

2. Vehicle and/or positive control: Fenoxycarb (750 mg a.s./L)

3. Test organism:

Species: *Apis mellifera*

Age: Not stated

Source: UK [redacted]

Acclimatisation: not required

4. Test system: Single chamber wooden Smith hive with British Standard frames. Twenty standardised field colonies housed in a single chamber wooden Smith hive with British standard frames each and headed by queens of similar age. The honey bee colonies contained 12000 – 22500 adult bees and consisted of 3-6 frames of brood (with few exceptions), 0.5-2 frames of honey and 0-1 frame of pollen.

Crop cultivated: Not applicable; the test site with no nearby flowering crops and few flowering weeds, [redacted] U.K.

Replication: 4 colonies/treatment and control

5. Environmental conditions:

Temperature: 3.4 – 46.3 °C

Relative humidity: 0 – 100%

Average wind speed: 4.0 – 13.1 mph

Precipitation: 0.0 – 9.71 mm

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B: STUDY DESIGN AND METHODS

1. Experimental treatments:

Test system: Twenty standardised honey bee colonies, each equipped with a dead bee trap fitted to the front were used in this study. All colonies were placed on varroa floors and sticky inserts were placed on the trays to trap any fallen mites. Colonies were located on a test site at [redacted] and allowed to fly freely, there were no nearby flowering crops and few flowering weeds (clover). Colonies were placed in groups according to treatment and placed at least 20 m apart from each other.

Experimental design: Up to 24 hours prior to dosing, 100 brood cells containing eggs, 100 cells containing 1-2 day old larvae and 100 cells containing 3-4 day old larvae were selected in each colony and marked using the Oomen *et al.* (1992)[6] acetate overlay sheet method.

Test doses: Dose setting was based on measured residues achieved in a glasshouse residues study (IIA 8.3.1.2./01) after spray application onto *Phacelia* plants at 2.88 kg glyphosate a.e./ha. Considering that bee colonies used in the brood study may be up to 50% bigger than those used in the residue study, an additional calculation for the expected total daily intake of glyphosate residues was undertaken assuming that such colonies would collect 9 g pollen and 1944 mL nectar (see table below). Furthermore the determined residue content based on application of 2.88 kg a.e./ha was adjusted to reflect the lower application rate of 2.16 kg a.e./ha.

Table 8.7.4-1: Exposure assessment of a brood study colony to glyphosate under two scenarios used to establish test doses for use in the brood study

Scenario	Daily intake of glyphosate residues in nectar (1944 g nectar/d) [mg]	Daily intake of glyphosate residues in pollen (9 g pollen/d) [mg]	Total daily intake of glyphosate residues [mg]	Uptake over 3 days [mg]	Adjustment from 2.88 kg a.e./ha to 2.16 kg a.e./ha [mg] [7]
Day 1 maximum mean residues (31.3 µg a.e./g in nectar, 574 µg a.e./g in pollen)	60.8 [1]	5.2 [2]	66.0	198	148.5 [3]
Mean residues over days 1-3 (15.5 µg a.e./g in nectar, 310 µg a.e./g in pollen)	30.3 [4]	2.8 [5]	33.1	99.3	74.5 [6]

[1] Derived from 1.944 kg nectar consumed/day * 31.3 mg/kg = 60.8 mg glyphosate a.e.
[2] Derived from 0.009 kg pollen consumed/day * 573.5 mg/kg = 5.2 mg glyphosate a.e.
[3] Value of 148.5 mg was rounded to 150 mg to achieve the nominal mid-dose concentration in brood study
[4] Derived from 1.944 kg nectar consumed/day * 15.6 mg/kg = 30.3 mg glyphosate a.e.
[5] Derived from 0.009 kg pollen consumed/day * 310.1 mg/kg = 2.8 mg glyphosate a.e.
[6] Value of 74.5 was rounded to 75 mg to achieve the nominal low-dose concentration in brood study
[7] The determined residue content based on application of 2.88 kg a.e./ha was adjusted to reflect the lower application rate of 2.16 kg a.e./ha.

Test item application: Three groups of colonies (i.e. four colonies per group) were treated with glyphosate isopropylamine salt added to 1 litre of 50% sucrose solution to achieve doses of 75, 150, and 301 mg a.e./L and one group was an untreated control, i.e. fed 1 litre 50% sucrose solution, only. In addition, one group was treated with the toxic reference fenoxycarb, dispersed in 1 L of 50% sucrose (750 mg a.s./L). Doses were administered by removing frames of stores from the colonies and placing a 1 litre glass container containing the treatment solution within the brood chamber.

[6] Oomen, P. A., De Ruijter, A., & Van der Steen J. (1992) Method for honeybee brood feeding tests with insect growth-regulating insecticides. Bulletin OEPP/EPPO Bulletin 22, 613-616.

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2. Observations: The content of dead bee traps was counted daily during the brood assessment period. All colonies were assessed within one week prior to dosing and within weeks 1, 2 and 3 after dosing, including counts of the number of combs of adults, brood, stores and pollen as well as behavioural or physical abnormalities. The uptake of each sucrose solution was checked daily and the container removed when empty or after 5 days. On day 7 the marked brood cells (eggs, young and old larvae) were assessed for mortality and appearance. On day 13 brood cells marked as containing old larvae, on day 15 cells previously containing young larvae and on day 16 cells previously containing eggs, were assessed. Cells were uncapped; the bee removed carefully with forceps and the age of bee was assessed, weighed and observed for deformities. The temperature and humidity were recorded continuously using a data logger; local (within 10 km) weather data was also collected.

3. Residues analysis: Analysis of glyphosate acid in larvae samples was conducted following extraction with acetonitrile:water (1:4, v/v), clean up by solid phase extraction on C18 and derivatisation as FMOC-glyphosate and a second clean up (solid phase extraction on Oasis HLB, methanolic elution) by HPLC-MS/MS. Analysis of glyphosate acid in treated sugar solution samples was conducted following extraction with acetonitrile:water (1:4, v/v), solid phase extraction on Oasis HLB, methanolic elution and derivatisation as FMOC-glyphosate by HPLC-MS/MS. Limit of quantification (LoQ) and limit of detection (LoD) were 1.0 and 0.3 mg/kg, respectively. Freshly prepared test treated sucrose solution samples were retained for analysis. On day 4 and 7, samples of ten 4-5 day old larvae were collected from each colony for residue analysis.

4. Data analysis: Brood mortality was analysed using a generalised linear model (Logit distribution) and an ANOVA for pupae weight data to determine NOAEL statistically.

II. RESULTS AND DISCUSSION

A. FINDINGS

Analytical data: Residues in samples of sucrose treatment solutions were within 11% of nominal doses. The nominal dose of 75 mg glyphosate a.e./L (corresponding to 61 mg glyphosate a.e./kg) was confirmed to be 65.7 mg glyphosate a.e./kg. The nominal dose of 150 mg glyphosate a.e./L (corresponding to 122 mg glyphosate a.e./kg) was confirmed to be 135 mg glyphosate a.e./kg. The nominal dose of 301 mg glyphosate a.e./L (corresponding to 245 mg glyphosate a.e./kg) was confirmed to be 266 mg glyphosate a.e./kg. (Conversion from nominal dose rate in mg a.e./L to nominal dose rate in mg/kg was based on a density of 50% w/v sucrose solution of 1.23 kg/L.)

Residues in larvae sampled from the hive on day 4 and day 7 ranged from 7.9 to 18.4 and below LOQ to 3 mg glyphosate a.e./kg, respectively on the dose 75 mg a.e./L, from 26.3 to 53.2 and 1.9 to 4.9 mg glyphosate a.e./kg, respectively on the dose 150 mg a.e./L and from 33.1 to 82.1 and 3.2 to 6.3 mg glyphosate a.e./kg, respectively on the dose 301 mg a.e./L, confirming that larvae were exposed to the test item provided in the sugar solution and consumed it.

B. OBSERVATIONS

Consumption of treated sucrose solution: The control colonies consumed between 0.625 and 1.0 L of untreated sucrose. In the glyphosate treated colonies at least 3 of 4 colonies consumed the total volume of treated sucrose.

Bee brood assessments:

Table 8.7.4-2: Survival of marked brood exposed to glyphosate isopropylamine salt

Dose rate [mg/L]	Control	75	150	301
Mean dose consumed [mg]		**73±2**	**138±12**	**255±46**
7-d old cells marked as eggs [%]	87.3±1.9	84.8±4.0	87.5±2.7	86.2±3.3
16-d old cells marked as eggs [%]	85.0±2.0	82.3±3.3	86.8±2.7	84.2±3.9
7-d old cells marked as young larvae [%]	96.4±3.0	93.5±1.8	91.5±4.3	95.0±1.8
16-d old cells marked as young larvae [%]	95.9±3.1	93.5±1.8	86.5±4.3	90.0±5.4
7-d old cells marked as old larvae [%]	97.0±0.4	96.8±0.5	96.8±1.7	95.3±2.9
16-d old cells marked as old larvae [%]	95.8±1.3	94.8±1.1	94.3±1.0	95.3±2.9

No significant statistical difference in brood development (eggs, young larvae, old larvae) was observed for all glyphosate treatment groups compared to control ($p<0.05$).

Table 8.7.4-3: Pupae weight at final assessment

Dose rate [mg/L]	Control	75	150	301
Mean dose consumed [mg]		**73±2**	**138±12**	**255±46**
Pupae marked as eggs [mg]	127.5±0.7	124.7±0.8	126.7±0.6	135.7±0.6
Pupae marked as young larvae [mg]	128.4±0.6	128.3±1.0	124.4±0.8	125.4±0.6
Pupae marked as old larvae [mg]	128.9±0.4	121.2±0.5	122.6±0.5	125.6±0.4

There were no significant effects of the treatment on the mean weight of the exposed pupae. No biologically significant adult mortality was observed in any treatment group. No adverse effects on colonies were observed in any treatment group apart from an apparent decline in the number of bees and brood in the fenoxycarb treated colonies in the later stages of the study.

In the fenoxycarb toxic reference treated colonies, the overall survival of marked cells was 20% for marked eggs, 0% for marked young larvae and 12% for marked old larvae, meeting the validity criterion for the toxic reference (>40% effect on all stages).

III. CONCLUSION

The overall NOAEL for brood development of honey bees was the highest dose tested – 301 mg glyphosate acid equivalent/L (nominal) equivalent to 245 mg glyphosate acid equivalent/kg nominal when considering the density of the sucrose solution and 266 mg glyphosate acid equivalent/kg actually measured.

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IIA 8.8 Effects on other arthropods

Existing data on non-target arthropods were assessed during the 2001-EU Evaluation and are summarised in SANCO/6511/VI/99-final. The data reviewed in the 2001 EU glyphosate evaluation are largely outdated Tier 1 studies and not acceptable for risk assessment according to the ESCORT 2 guidance.

The effects of the original Roundup herbicide formulation have been investigated in the screening level assay with 18 different beneficial predators and parasites (Hassan et al. 1988)[7]. Roundup was found to be harmless to thirteen species, slightly harmful to four species and moderately harmful to one species of carabid beetles. The authors did not believe that sufficient toxicity potential existed to warrant semi-field and field tests that were performed on some of the other compounds tested in the same program.

Additional studies with the lead formulation MON 52276 have been conducted with *Aphidius rhopalosiphi*, *Typhlodromus pyri* and *Aleochara bilineata* to meet the data requirements outlined in the ESCORT2 guidance document that are acceptable for an updated non-target arthropod risk assessment. A summary of data for MON 52276 reviewed in the 2001 EU glyphosate evaluation and the recently performed studies with the lead formulation are summarized in Table 8.8-1.

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[7] Hassan, et al. 1988. Results of the fourth joint pesticide testing programme carried out by the IOBC/WPRS Working Group "Pesticides and Beneficial Organisms". J.APPL., 1988, 321~329.

Table 8.8-1: Toxicity of MON 52276 to terrestrial non-target arthropods other than bees

Species	Test type	Treatment rates (L/ha)	Results	Reference/GLP	2001 EU evaluation monograph reference
MON 52276					
Typhlodromus pyri	Laboratory	**10**	**100% mortality**	95 10 48 056 1995/yes	
Typhlodromus pyri	Extended laboratory (leaf discs)	**> 75% mortality at 6 and 12 L/ha; 36% mortality at 3 L/ha; No effects on fecundity at 3 L/ha after 16 d**		-98-195 , 1998/yes	
Typhlodromus pyri	Extended laboratory (leaf discs)	No effect on mortality at 16 L/ha No effects on fecundity at 8 L/ha		IIA 8.8.2.2/01 -2009-404 2010/yes	
Typhlodromus pyri	Extended laboratory (whole leaf)	**36% and 30% mortality at 6 and 12 L/ha, respectively; 21% mortality at 3 L/ha; No mortality at 0.6 L/ha; No effects on fecundity at 12 L/ha or less**		-99-092 1999/yes	
Aphidius rhopalosiphi	Laboratory	**10**	**100% mortality**	95 10 48 054 1995/yes	
Aphidius rhopalosiphi	Extended laboratory (whole plant)	**No effect on mortality at 12 L/ha No effects on fecundity at 12 L/ha**		98 10 48 066 1999/yes	
Aphidius rhopalosiphi	Extended laboratory (whole plant)	No effect on mortality at 16 L/ha No effects on fecundity at 16 L/ha		IIA 8.8.2.1/01 -2009-405 2010/yes	
Chrysoperla carnea	Laboratory	**59% mortality and 20% reduction on fecundity at 12 L/ha (no dose-response effect on fecundity); No effects on survival or fecundity at 0.6 and 6 L/ha**		-99-3 1999/yes	
Aleochara bilineata	Extended Laboratory (soil)	No effect on mortality at 12 L/ha No effects on fecundity at 12 L/ha		IIA 8.8.2.3/01 -2009-403 2010/yes	
Bembidion lampros	Semi-field	**4.89 kg a.s./ha**	**0% adult mortality**	1990	ANA 96-00074
Poecilus cupreus	Laboratory	**10**	**No effects on survival or feeding activity**	-2000-203 1995/yes	
Trechus quadristriatus	Laboratory	**3.6 kg a.s./ha**	**14% adult mortality**	1990	ANA 96-00072
Pardosa sp.	Laboratory	**10**	**No effects on survival or feeding activity**	-2000-204 1995/yes	

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

The toxicity of MON 52276 to the parasitoid *Aphidius rhopalosiphi*, as would be expressed under field conditions, is expected to be low. Although effects were seen in a Tier 1 test with the parasitoid *Aphidius rhopalosiphi* following exposure to fresh dried residues applied at 10 L/ha (3.6 kg a.s./ha) onto a glass surface, it was noted that there appeared to be formulation effects, with the wasps affected by sticky deposits on the glass. When an extended laboratory test was conducted using exposure to fresh dried residues applied at 12 L/ha (4.32 kg a.s./ha) onto a realistic substrate (a plant surface), there were no significant effects (mortality and parasitation were < 30% compared to the controls), indicating that under realistic exposure conditions, significant effects are unlikely to occur.

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Artificial exposure conditions also resulted in increased toxicity of MON 52276 to the predatory mite *Typhlodromus pyri*. Significant effects (100% mortality) on the predatory mite *Typhlodromus pyri* were seen in a Tier 1 test following exposure to fresh dried residues of MON 52276 applied at a rate of 10 L/ha (3.6 kg a.s./ha) onto a glass surface. Two extended laboratory tests were conducted with exposure to fresh dried residues on a realistic substrate (on a leaf surface or on a leaf on a living plant), and demonstrated a lower mortality than the Tier 1 test. In the test conducted on a living plant, mortalities of 36 and 30% were observed at 6 and 12 L/ha, respectively. At 3 L/ha, 21% mortality was observed. No effects on fecundity were observed at rates up to 12 L/ha in the living plant test. These results indicate that, under realistic exposure conditions, significant effects are unlikely to occur.

While mortality was observed in a laboratory test with the green lacewing (*Chrysoperla carnea*) following exposure to fresh dried residues of MON 52276 applied at a rate of 12 L/ha (4.32 kg a.s./ha) onto a glass surface. However, no effects on mortality or fecundity were observed at 0.6 and 6 L/ha, and effects on fecundity at the rate of 12 L/ha were only 20%.

The results of the laboratory beneficial arthropod studies indicate that the toxicity of MON 52276 to the representative ground-dwelling non-target arthropods carabid beetle *(Poecilus cupreus)* and lycosid spider *(Pardosa spp.)* is low, even when tested under worst-case (Tier 1) laboratory conditions with direct and residual exposure at 10 L/ha using an inert substrate (quartz sand).

Non-target arthropod studies on the active substance which were not included in the previous EU-Evaluation of glyphosate (2001) or SANCO 6511/VI/99-final are summarised below under the corresponding Annex III points.

IIA 8.8.1 Effects on non-target terrestrial arthropods using artificial substrates

Data on non-target arthropods using artificial substrates were assessed during the 2001-EU Evaluation and are summarised are summarized in Table 8.8-1.

IIA 8.8.1.1 Parasitoid

For effects of MON 52276 on *Aphidius rhopalosiphi* using artificial substrates please refer to Table 8.8-1.

IIA 8.8.1.2 Predatory mites

For effects of MON 52276 on *Typhlodromus pyri* using artificial substrates please refer to Table 8.8-1.

IIA 8.8.1.3 Ground dwelling predatory species

For effects of MON 52276 on *Poecilus cupreus*, *Trechus quadristriatus* and *Pardosa* ssp. using artificial substrates please refer to Table 8.8-1.

IIA 8.8.1.4 Foliage dwelling predatory species

For effects of MON 52276 on *Chrysoperla carnea* using artificial substrates please refer to Table 8.8-1.

IIA 8.8.2 Effects on non-target terrestrial arthropods in extended laboratory/semi field tests

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IIA 8.8.2.1 Parasitoid

Annex point	Author(s)	Year	Study title
IIA 8.8.2.1/01	[redacted]	2010	A rate-response extended laboratory test to determine the effects of MON 52276 on the parasitic wasp, *Aphidius rhopalosiphi* (Hymenoptera, Braconidae) [redacted] Report No: [redacted]-09-2 ([redacted]-2009-405) Date: 2010-04-27 GLP: yes not published

Guideline: Mead-Briggs *et al.* (2010). An extended laboratory test for evaluating the effects of plant protection product on the parasitic wasp, *Aphidius rhopalosiphi* (De Stefani-Perez) (Hymenoptera, Braconidae).

Deviations to Mead-Briggs et al. (2010): None

Dates of experimental work: 2009-10-14 to 2009-11-09

Executive Summary

The toxicity of MON 52276 to the parasitic wasp, *Aphidius rhopalosiphi* was determined in an extended laboratory test. Adult parasitic wasps approximately 48 h old were exposed in a definitive rate-response test to 4000, 6000, 8000, 12000 and 16000 mL product/ha (nominally 1440, 2160, 2880, 4320, and 5760 g a.e./ha). In addition, a water control (negative control) and a toxic reference ([redacted], 400 g/L dimethoate) were tested.

Treatments were applied at a volume rate equivalent to 400 L spray solution/ha to pots of seedling barley. Once dry, the barley plants were enclosed within cylindrical, ventilated collars. Five female wasps were exposed per replicate, with six replicates (i.e. a total of 30 wasps) prepared for each treatment. Mortality and sublethal effects were recorded 2 (3), 24 and 48 hours after application.

To assess any significant sub-lethal effects, reproduction assessments were then carried out for the highest three treatment rates of the test item that resulted in < 50% mortality and from the control. Up to 15 female wasps were confined individually for 24 h over untreated barely plants infested with the cereal aphids, *Rhopalosiphi padi* (L.) and *Metopolophium dirhodum* (Walk). The wasps were then removed and the plants were left for a further 10 days before the number of aphid mummies that had developed was recorded.

The validity criteria according to Mead-Briggs *et al.* (2010) are fulfilled.

In conclusion, in an extended laboratory test to determine the effects of MON 52276 on the parasitic wasp, *Aphidius rhopalosiphi*, the 48 h LR_{50} was higher than 16000 mL product/ha. MON 52276 had no adverse effects on reproductive performance of surviving wasps up to and including a treatment rate of 16000 mL product/ha.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 52276
Description: Not stated
Lot/Batch #: A9B1207115
Purity: Glyphosate (isopropylamine salt) 360 g/L
Density: 1.1683 g/cm^3 (at 20 °C ± 0.5 °C)

2. Vehicle and/or positive control: [redacted] (dimethoate: 400 g/L)

3. Test organisms:

Species: Parasitic wasp (*Aphidius rhopalosiphi*)
Age: Adults approximately 48 h old
Source: [redacted]
[redacted] Germany
Diet/Food: Solution of honey in water (1 : 3 v/v)

4. Environmental conditions:

Temperature: 20 °C
Relative humidity: 69 – 72 %
Photoperiod: 16 hours light / 8 hours darkness

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Following a preliminary range-finding test, MON 52276 was evaluated in a definitive rate-response test at five application rates, equivalent to 16000, 12000, 8000, 6000 and 4000 mL product/ha. These variants were compared to a control treatment of purified water and a toxic reference treatment of [redacted] (nominally 400 g/L dimethoate) applied at a rate of 10 mL product/ha (nominally 4 g a.s./ha).Treatments were applied at a volume rate equivalent to 400 L spray solution/ha to pots of seedling barley. Once dry, the barley plants were enclosed within cylindrical, ventilated collars. Five female wasps were then confined in each arena, with six replicates (i.e. a total of 30 wasps) prepared for each treatment. To determine any significant sub-lethal effects on wasp reproduction, assessments were then carried out using the surviving insects from the control and the three highest treatment rates of the test item that resulted in ≤ 60% corrected mortality. Fifteen wasps from each treatment were confined individually over pots of untreated barley plants that had previously been infested with cereal aphids (*Metopolophium dirhodum* and *Rhopalosiphum padi*). The wasps were then removed from the plants after 24 hand the aphids and plants left for a further 10 days before the number of 'mummies'(parasitized aphids containing wasp pupae) that had developed was recorded.

2. Observations: Mortality of the wasps was recorded approximately 2, 24 and 48 h after treatment. The behaviour of the wasps was assessed during the first 3 h after treatment and also at 24 and 48 h after treatment, to determine whether there was any apparent repellence from the treated plants, and wasp survival was assessed over a period of 48 h.

3. Statistical calculations: ANOVA followed by Fisher's Exact test ($\alpha = 0.05$) for mortality. One-way ANOVA and Dunnett's Test as post hoc ($\alpha = 0.05$) for reproduction.

II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

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Table 8.8-2: Toxicity of MON 52276 to parasitic wasps (*Aphidius rhopalosiphi*) in a 48 h extended laboratory test

Test rate [mL/ha]	Mortality [%]	Corrected mortality [%] [1]
Control	0	-
4000	0	0
6000	0	0
8000	0	0
12000	3.3	3.3
16000	0	0

1 Derived using Abbott's formula.

Reference test: Treatment with the reference item [redacted] at a concentration of 10 mL/ha resulted in 90% mortality after 48 h of exposure.

Table 8.8-3: Sublethal effects of MON 52276 to parasitic wasps (*Aphidius rhopalosiphi*) in a 48 h extended laboratory test (summary of wasp repellence assessments)

Test rate [mL/ha]	% observations where wasps recorded to be settled on the treated plants	
	Initial 3 h [1)]	24 h & 48 h [2)]
Control	32.7	40.0
4000	22.0	28.3
6000	24.7	28.3
8000	26.0	25.0
12000	20.7 *	27.5
16000	20.0 *	28.3

1 Data from assessments made during the initial 3 h after wasp introduction. Results for the individual test item treatments were compared by one-way ANOVA and Dunnett's Test. Values marked with asterisks differed significantly from the control (* $P < 0.05$).

2 Data from assessments made at 24 h and 48 h after wasp introduction. Results for the individual test item treatments were compared by one-way ANOVA ($\alpha = 0.05$), but values for the test item treatments did not differ significantly from the control.

Reference test: Treatment with the reference item [redacted] at a concentration of 10 mL/ha resulted in significant effects on reproduction after 48 h of exposure.

The mortality in the control treatments did not exceed 10%, the corrected mortality in the reference treatment was >50%. In the control treatments, more than a minimum mean value of 5.0 mummies was produced per female. Not more than two of the surviving wasps of the control treatments did not reproduce. Therefore, the test is considered valid according to Mead-Briggs *et al.* (2010).

III. CONCLUSION

In an extended laboratory test to determine the effects of MON 52276 on the parasitic wasp, *Aphidius rhopalosiphi*, the 48-h LR_{50} was higher than 16000 mL product/ha. MON 52276 had no adverse effects on the reproductive performance of surviving wasps up to and including a treatment rate of 16000 mL product/ha.

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IIA 8.8.2.2 Predatory mites

Annex point	Author(s)	Year	Study title
IIA 8.8.2.2/01	[redacted]	2010	An extended laboratory bioassay of the effects of fresh residues of MON 52276 on the predatory mite, *Typhlodromus pyri* (Acari: Phytoseiidae) [redacted] [redacted] Report No: [redacted]-2009-404 Date: 2010-04-27 GLP: yes not published

Guideline: IOBC/BART/EPPO (Blümel et al., 2000)

Deviations to Barrett et al. (1994): It was intended that the bioassay would take place in a cabinet maintained at 60-90% RH, although minor fluctuations outside of these parameters for periods of <2h were con to be considered as deviations (according to the guideline of Blümel et al., 2000). However, in the range-finding bioassay the ambient conditions actually recorded were 51.9%-61.4% RH. These deviations were due to inadequate control being achieved by the cabinet. Since all treatments were exposed to similar conditions (and this was in the range-finding bioassay), it was considered that this deviation did not affect the outcome of the bioassay, nor the integrity of the study.

Dates of experimental work: 2009-10-19 to 2009-11-24

Executive Summary

The toxicity of MON 52276 to the predatory mite (*Typhlodromus pyri*) was determined in an extended laboratory test. MON 52276 was evaluated at five rates, equivalent to 16000, 12000, 8000, 6000, 3000 mL formulation/ha (nominally 5760, 4320, 2880, 2160, and 1080 g a.s/ha). These were compared to a control treatment of deionised water (negative control) and a toxic reference treatment of [redacted] (nominally 400 g/L dimethoate) applied at a rate of 30 mL formulation/ha (nominally 12 g a.s./ha). Treatments were applied to 5-cm-diameter leaf discs (n = 3 per treatment) cut from French bean plants (*Phaseolus vulgaris* L.). Once residues had dried, a ring of sticky gel was drawn on each of the leaf discs to create arenas in which mites were then confined. Twenty protonymphal *T. pyri* were placed in each replicate unit, with three replicates (i.e., 60 mites) prepared per treatment. The mites were provided daily with untreated almond and apple pollen for food and their survival was assessed at 1 and 7 days after treatment, by which time they were adult. Assessments were then made of the reproductive capacity of the mites surviving in the control treatment and in all test item treatments in which < 50% corrected mortality had been observed. The sex of the mites was determined and they were left on the leaf discs, with untreated pollen being provided for food daily. Egg production was assessed at 10, 13, and 14 days after treatment (DAT) and the mean number of eggs produced per female was calculated. The 7-day LR_{50} (median lethal rate) was found to be higher than 16000 mL formulation/ha (nominally 5760 g a.e./ha). MON 52276 had no adverse effects on the reproductive performance of surviving mites up to and including a treatment rate of 8000 mL formulation /ha (nominally 2880 g a.e./ha).

In conclusion, the effects of MON 52276 on the predatory mite, (*Typhlodromus pyri*) were evaluated under extended laboratory test conditions. The 7-day LR_{50} (median lethal rate) was found to be higher than 16000 mL/ha (nominally 5760 g a.e./ha), the maximum rate tested. MON 52276 had no significant effect on the reproductive capacity of mites at treatment rates up to and including a treatment rate of 8000 mL formulation/ha (nominally 2880 g a.e./ha).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 52276
Description:	Yellow/amber fluid
Lot/Batch #:	A9B1207115
Purity:	31.92 % w/w glyphosate acid , measured
2. Vehicle and/or positive control:	[redacted] Dimethoate 40 (400 g/L dimethoate)
3. Test organisms:	
Species:	Predacious mite (*Typhlodromus pyri*)
Age:	< 24 hours
Source:	[redacted] Germany
Diet/Food:	Almond and apple pollen
Acclimatisation:	Not stated
4. Environmental conditions:	
Temperature:	Mortality test: 25- 26°C Reproductive test: 25- 27 °C
Relative humidity:	Mortality test: 49.6 – 79% Reproductive test: 63 - 79%
Photoperiod:	16 hours light / 8 hours darkness
Light intensity	660 – 1230 lux

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: Leaf discs (5-cm diameter) cut from detached primary leaves of bean plants (*Phaseolus vulgaris* L.) were sprayed with MON 52276 at rates equivalent to 16000, 12000, 8000, 6000, and 3000 mL/ha (5760, 4320, 2880, 2160, and 1080 g a.e./ha). Additional leaf discs were treated with the reference toxicant [redacted] (414.8 g dimethoate/L) applied at a rate of 12 g a.s./ha, or with deionised water as a negative control. All treatments were sprayed in an application volume equivalent to 200L/ha. After the sprayed discs were air-dried for approximately 1 hour, protonymphal mites were placed on the leaves, inside petri dishes, and observed for seven days. There were three replicates, each containing twenty mites, per treatment. On Day 7, any eggs present were removed, and the number of male and female mites in each of the replicates was recorded to verify the male:female ratio was at least 1:5. Between days 7 and 14, a fecundity assessment was carried out for the control and the treatments. The nymphs were fed with a mixture of almond and apple pollen provided daily, and were provided water *ad libitum* throughout the study. Test units were kept under a 16-hour:8-hour light:dark regimen.

2. Observations: The number of living, dead, stuck, drowned, or missing mites was recorded on Days 1 and 7. The number of eggs laid, and live or dead juvenile states per female were recorded on days 10, 13, and 14.

3. Statistical calculations: The mortality was corrected with the control mortality using Abbot's correction (1925).The percentage mortality in each treatment in the bioassay was compared to the control

using Fisher's Exact Test ($\alpha = 0.05$). Reproduction results were compared by one-way ANOVA and Dunnett's Test.

II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

The test item resulted in 40% mortality of *Typhlodromus pyri* when applied at concentration of 16000mL/ha and higher. In the fecundity assessment, no dose-response relationship was observed.

Table 8.8-4: Toxicity of MON 52267 to predatory mites (*Typhlodromus pyri*) in a 7 d laboratory test

Test concentration [L/ha]	Mortality after 7 days [%][1]	Abbott corrected mortality [%][2]	Mean egg number/ female after 14 days	Effects on Reproduction[3]
Control	15	-	6.9	-
16000	40*	29	3.0*	56.5
12000	32	20	3.8*	44.9
8000	23	9	5.9	14.5
6000	18	4	4.2	39.1
3000	13	0	8.1	-17.4
Toxic reference (, 12 g a.s./ha)	87*	85	-	-

1 Including dead, stuck, drowned, or missing mites.
2 According to Abbott (1925)
3 Change in numbers of eggs per female relative to control (after Blümel et al., 2000). A positive value indicates a decrease and a negative value indicates an increase.
* Significantly different from control ($\alpha = 0.05$)

Reference test: Treatment with the reference item Dimethoate 40 resulted in significant effects on reproduction (87%).

Validity criteria according to Candolfi et al. (2000) were fulfilled; mortality in control group did not exceed20%, the mortality in the toxic reference treatment was between 50-100%, and the mean cumulative umber of eggs produced between 7 and 14 days exceeded 4.0 per female in the control treatment.

III. CONCLUSION

The effects of MON 52276 on the predatory mite, (*Typhlodromus pyri*) were evaluated under extended laboratory test conditions. The 7-day LR_{50} (median lethal rate) was found to be higher than 16000 mL/ha (nominally 5760 g a.e./ha), the maximum rate tested. MON 52276 had no significant effect on the reproductive capacity of mites at treatment rates up to and including a treatment rate of 8000 mL formulation/ha (nominally 2880 g a.e./ha).

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IIA 8.8.2.3 Ground dwelling predatory species

Annex point	Author(s)	Year	Study title
IIA 8.8.2.3/01	███	2010	An extended laboratory test to determine the effects of MON 52276 on the ground-active beetle, *Aleochara bilineata* (Coleoptera, Staphylinidae) Report No: ███-09-4 (███-2009-403) Date: 2010-04-27 GLP: yes not published

Guideline: Grimm *et al* (2000). A test for evaluating the chronic effects of plant protection products on the rove beetle, *Aleochara bilineata* Gyll. (Coleoptera: Staphylinidae), under laboratory and extended laboratory conditions

Deviations to Grimm et al. (2000): None

Dates of experimental work: 2009-10-02 to 2010-01-02

Executive Summary

In the extended laboratory study the toxicity of MON 52276 to the rove beetle, *Aleochara bilineata* was tested. Adult rove beetles (3 - 4 days old) were exposed in the definitive rate-response test to 6000, 8000 and 12000 mL product/ha. In addition, a water control (negative control) and a toxic reference (███, 480 g/L chlorpyrifos) were tested.

Ten female and ten male beetles were introduced in each testing arena, with two replicates (i.e. a total of 20 beetles) prepared for each treatment. Assessments of the condition of the beetles were made at 1, 7 and 28 days after treatment (DAT).The parasitic success of their larval offspring was assessed by the provision of ca. 500 onion fly pupae (*Delia antiqua*) in each replicate box on three weekly occasions, i.e. at 7, 14 and 21 DAT. The original adult beetles were removed from the arenas at 28 DAT and the number of new adults (F1 progeny) that subsequently developed from the parasitized fly pupae was recorded over a further 46-day period. The validity criteria according to Grimm *et al.* (2000) are fulfilled.

In conclusion, in the extended laboratory test to determine the effects of MON 52276 on the rove beetle (*Aleochara bilineata*), no significant effect on the parasitation success of the beetles were observed up to and including the highest treatment rate of 12000 mL/ha.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 52276
Description: Yellow/amber-coloured liquid appearance
Lot/Batch #: A9B1207115
Purity: Glyphosate (glyphosate acid equivalent) 360 g/L
Density: 1.1683 g/cm^3 (at 20 °C ± 0.5 °C)

2. Vehicle and/or positive control: Reference item: ███ (chlorpyrifos: 480 g/L)

3. Test organisms:

Species: Rove beetle (*Aleochara bilineata*)

Age: Physiologically 3 - 4 days old

Source: Netherlands)

Diet/Food: Pellets (approximately 0.2-0.5 g) of raw minced beef for food every 1-3 days, until the adult beetles were removed 28 days after treatment (DAT)

Acclimatisation: Not stated

4. Environmental conditions:

Temperature: 19 – 21 °C

Relative humidity: 51 – 86 %

Photoperiod: 16 hours light / 8 hours darkness

B: STUDY DESIGN AND METHODS

1. Experimental treatments: MON 52276 was evaluated at three treatment rates, equivalent to 6000, 8000 and 12000 mL product/ha. These were compared to a water-treated control (negative control) and a toxic reference treatment of chlorpyrifos (a 480 g/L EC formulation applied at a rate equivalent to 240 g a.s./ha). All treatments were applied to boxes of a standard sandy soil (LUFA 2.1), using a track sprayer calibrated to deliver the equivalent of 400 L spray solution/ha. Applications were made to four replicate arenas per treatment and, immediately following spraying, twenty adult *Aleochara bilineata* (10 males: 10 females) were introduced into each replicate. Beetles were fed with raw minced beef, initially an hour after treatment and then every 2 to 3 days thereafter. The parasitic success of their larval offspring was assessed by the provision of ca. 500 onion fly pupae (*Delia antiqua*) in each replicate box on three weekly occasions, i.e. at 7, 14 and 21 DAT. The original adult beetles were removed from the arenas at 28 DAT and the number of new adults (F1 progeny) that subsequently developed from the parasitized fly pupae was recorded over a further 46-day period.

2. Observations: Assessments of the condition of the beetles were made at 1, 7 and 28 days after treatment (DAT).Assessment of reproduction was conducted from 28 DAT for 46 days.

3. Statistical calculations: Fisher's Exact test ($\alpha = 0.05$) for mortality, ANOVA ($\alpha = 0.05$) for reproduction.

II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

Mortality

Table 8.8-5: Toxicity of MON 52276 to rove beetles (*Aleochara bilineata*) after 28 days in an extended laboratory test

Test rate [mL/ha]	Mortality [%]	Corrected mortality [%] [1]
Control	32.5	-
6000	38.8	9.3
8000	47.5	22.2
12000	38.8	9.3

[1] Derived using Abbott's formula

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Reference test: Treatment with the reference item [redacted] at a concentration of 240 g a.s./ha resulted in 100% mortality after 28 d of exposure.

Table 8.8-6: Sublethal effects of MON 52276 to rove beetles (*Aleochara bilineata*) in an extended laboratory test (mean number of F1 progeny)

Test rate [mL/ha]	Mean number of F_1 progeny per arena [1]	Standard deviation	Effect on reproduction [%] [2]
Control	862.5	66.8	--
6000	706.3	84.6	18.1
8000	846.0	109.5	1.9
12000	778.0	102.6	9.7

[1] The numbers of progeny emerging in the control and test item treatments were compared by ANOVA, but treatment means did not differ significantly ($P > 0.05$). For the toxic reference treatment (where all values were zero), no statistical comparisons were made.

[2] The percentage change in numbers of F1 progeny, relative to the control was calculated using the formula: $R = (1-(Rt/Rc) \times 100$, where Rt and Rc are the numbers of offspring observed in the treatment and control groups, respectively. Positive values indicate a decrease, relative to the control.

Reference test: Treatment with the reference item [redacted] at a concentration of 240 g a.s./ha resulted in 100% effects on reproduction.

The average number of beetles emerging from parasitized fly pupae in the control treatment was >400 per replicate, and a minimum reduction of 50% reproductive capacity was achieved in the reference item treatment when compared to the control. The validity criteria according to Grimm *et al.* (2000) are therefore fulfilled.

III. CONCLUSION

In an extended laboratory test to determine the effects of MON 52276 on the rove beetle (*Aleochara bilineata*), no significant effect on the parasitation success of the beetles were observed up to and including the highest treatment rate of 12000 mL/ha.

IIA 8.8.2.4 Foliage dwelling predatory species

Extended laboratory or semi-field studies on foliage dwelling predatory species were not required according to Commission Document 76511/VI/99-final dated 21 January 2002 and Directive 2001/99/EC dated 20 November 2001.

IIA 8.8.2.5 Other terrestrial invertebrates

Extended laboratory or semi-field studies on other terrestrial invertebrates were not required according to Commission Document 76511/VI/99-final dated 21 January 2002 and Directive 2001/99/EC dated 20 November 2001.

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IIA 8.9 Effects on earthworms

In the 2001 EU Evaluation of Glyphosate it was concluded that acute and chronic risk to earthworms is considered low. New higher dose laboratory studies with the IPA-salt of glyphosate and AMPA confirm the low toxicity of glyphosate and its major soil metabolite AMPA on earthworm populations at field exposure concentrations. A summary of all available relevant and compliant data (including data already reviewed during the 2001 EU evaluation of glyphosate) for glyphosate, glyphosate salts and the metabolite AMPA is presented in Table 8.9-1.

Since the 2001-EU Evaluation, data requirements for other soil non-target macro-invertebrates have changed. Therefore, new studies have been conducted exposing *Hypoaspis aculeifer* and *Folsomia candida* to glyphosate IPA salt and AMPA, respectively (see Table 8.9-2). In the 200 EU glyphosate evaluation a review summarizing effects of glyphosate on soil fauna was reviewed (Eijsackers, 1985)[8]. This paper examined effects on isopods and litter decomposition. No decreases in litter consumption or dose-related adverse effects to macro-organism communities were found. The results of the new standard guideline studies with the IPA salt of glyphosate confirm the previous conclusion of the 2001 EU Glyphosate evaluation. These studies were conducted with the IPA salt as a representative salt for glyphosate based formulations and represent a realistic exposure to glyphosate under field conditions.

Table 8.9-1: Toxicity of glyphosate acid, IPA salt, its metabolite AMPA and formulation MON 52276 to earthworms

Species	Test design	LC_{50} (mg a.s./kg dry soil)	NOEC (mg a.s./kg dry soil)	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid					
Eisenia fetida	14 d acute	>1000	1000 [1]	[redacted] 502 [redacted] 1995/yes	ARW 96-00093
Eisenia fetida	14 d acute	>1000		-	ARW 96-00099 [redacted] 1990
Eisenia fetida	14 d acute	>1000	1000 [1]	-	ARW 96-00095 [redacted] 1995
Eisenia fetida	14 d acute	5600 – 10000	<5600	IIA 8.9.1/01 [redacted] -1875 [redacted] 2002/yes	-
Eisenia fetida	56 d chronic	-	16.8 [1]	[redacted] -1878 [redacted] 2002/yes Summary not included and can be provided upon request.	-
Glyphosate-IPA salt					
Eisenia fetida	14 d acute	>1000	1000 [1]	[redacted] -92-0024-01 [redacted] [redacted] 1992/yes	ARW 96-00091
Eisenia fetida	14 d acute	>5000	158.2	80-91-2078-06-91 [redacted], 1995/yes	ARW 96-00096

[8] Eijsackers, H. 1985. Effects of glyphosate on the soil fauna. In: The herbicide glyphosate, eds.: Grossbard, E., Atkinson, D., Butterworth, 1985, 151-158. L1435. ARW95-00109.

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Species	Test design	LC_{50} (mg a.s./kg dry soil)	NOEC (mg a.s./kg dry soil)	Reference/GLP	2001 EU evaluation monograph reference
Eisenia fetida	56 d chronic	>638.1	638.1 [1] 472.8 mg a.e./kg dry soil	IIA 8.9.2/01 09 10 48 056 S ███ 2009/yes	-
Eisenia fetida	56 d chronic	-	**28.79** [1] 21.31 mg a.e./kg dry soil	IIA 8.9.2/02 ███-1173 ███ ███ 2000/yes	-
AMPA					
Eisenia andrei	14 d acute	>1000	100	IIA 8.9.1/02 F13███ ███ 2000	-
Eisenia fetida	56 d chronic	-	28.12 [1]	IIA 8.9.2/02 ███-1173 ███ ███ 2000/yes	-
Eisenia fetida	56 d chronic	-	19.7 [1]	IIA 8.9.2/03 011120███ ███ 2000/yes	-
Eisenia fetida	56 d chronic	-	198.1	IIA 8.9.2/04 01-64-077-███ ███ 2003/yes	-
Eisenia fetida	56 d chronic	-	5.53 [1]	███-1878 ███ 2002/yes Summary not included and can be provided upon request.	-

[1] Highest concentration tested

Values in bold: confirmed EU endpoints (SANCO/6511/VI/99-final, or EU Review Monograph)

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Table 8.9-2: Toxicity of glyphosate IPA and its metabolite AMPA to soil macro organisms other than earthworms

Species	Test design	EC_{50} (mg a.s./kg dry soil)	NOEC (mg a.s./kg dry soil)	Reference/GLP
Glyphosate-IPA salt				
Hypoaspis aculeifer	14 d chronic	>1000 mg/kg [1] >472.8 mg a.e./kg	1000 mg/kg 472.8 mg a.e./kg	IIA 8.9.2/05 09 10 48 058 S ███ 2009/yes
Folsomia candida	28 d chronic	>1000 mg/kg [1] >587 mg a.e./kg	1000 mg/kg 587 mg a.e./kg	IIA 8.9.2/06 09 10 48 057 S ███ 2010/yes
AMPA				
Hypoaspis aculeifer	14 d chronic	>320 [1]	320	IIA 8.9.2/07 10 10 48 053 S ███, 2010/yes
Folsomia candida	28 d chronic	>315 [1]	315	IIA 8.9.2/08 10 10 48 054 S ███ 2010/yes

[1] EC_{50} values correspond to the highest dose tested.

Studies not submitted within the scope of the 2001 EU Evaluation of Glyphosate are summarised below. The endpoint for acute toxicity of glyphosate to earthworms of the previous 2001 EU Evaluation of Glyphosate (SANCO/6511/VI/99-final) was based on a limit test conducted with a 360 g a.s./L SL-formulation (LC_{50}> 480 mg a.s./kg; , 1991, ARW 96-00094) and can be safely replaced by a number of newer, more relevant studies conducted with the active substance and glyphosate-IPA salt, indicating that the relevant **LC_{50} for earthworms is >1000 mg a.s./kg dry soil.** To evaluate the endpoint for chronic toxicity of glyphosate and its metabolite AMPA further, since the 2001-EU evaluation of glyphosate additional studies have been performed and are summarised below.

IIA 8.9.1 Acute toxicity to earthworms

Annex point	Author(s)	Year	Study title
IIA 8.9.1/01		2002	Sinon Glyphosate Technical: The Acute Toxicity to the Earthworm *Eisenia foetida* Report No: -1875 Date: 2002-11-29 GLP: yes Not published

Guideline: OECD Guideline No. 207 (1984)

Deviations to OECD 207: none

Dates of experimental work: 2002-09-17 to 2002-11-01

Executive Summary

The effects of glyphosate acid on the earthworm *Eisenia fetida* were tested in a 14 day acute laboratory test. The endpoints that were evaluated were mortality, body weight, appearance and behaviour. The test was conducted with two concentrations in the definitive test (5600 and 10000 mg glyphosate acid /kg dry soil) and a negative control using OECD soil with 10% peat moss.

After 14 days, 32 out of 40 (80%) worms died at the highest tested concentration of 10000mg glyphosate acid equivalents. One earthworm (2.5%) died at 5600 mg a.e./kg dry soil) Furthermore, the average weights of the earthworms decreased significantly at 10000 mg a.e./kg dry soil. All validity criteria according to the OECD guideline 207 were fulfilled.

The LC_{50} of glyphosate acid is 5600 – 10000 mg a.e./kg soil. The NOEC for glyphosate acid was determined to be 5600 mg a.e./kg soil based on mortality and < 5600 mg a.e./kg soil based on weight loss.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

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Test item: Glyphosate acid
Description: White crystalline powder
Lot/Batch #: 20020727
Purity: 97.1 % w/w

2. Vehicle and/or positive control: Reference substance: 2-chloracetamide

3. Test organism:

Species: Earthworm (*Eisenia fetida*)
Age: Definitive test: adults, ca. 7 – 8 months old
Weight: 336 - 407 mg (test initiation)
Source:
Food: None
Acclimation period: no data

4. Environmental conditions:

Temperature: 18.6 – 20.5 °C (treatments), 19.0 – 20.5 °C, measured on day 0, 7 and 14
Photoperiod: 24 h light (mean 696lux, range: 581 – 787 lux), measured on day 0, 7, 14
Soil pH: 3.71 – 4.40 (treatments); 5.50 – 5.54 (control)
Soil moisture content: 30 - 35 %

B. STUDY DESIGN AND METHODS

1. Experimental treatments: Since in a range finding test no mortality was observed at 10000 mg a.s./kg dry soil, the main test was conducted with only two test concentrations,5770 and 10300 mg glyphosate acid/kg dry soil (equivalent to 5600 and 10000 mg a.e./kg dry soil) and a negative control was performed in parallel. The test item was mixed into the artificial soil substrate (10% Sphagnum-peat; 20% kaolin clay, 70% industrial quartz sand, pH adjusted to 5.5 – 6.5 with calcium carbonate). The moisture content was adjusted to about 35% using deionised water. Four replicate test containers (1000 mL glass beakers covered with polyethylene adhesive film and perforated with holes) during the definite test, were prepared for each treatment group. 10 adult earthworms per replicate were exposed for 14 days. The negative control was treated with deionised water only. As a toxic reference, earthworms were exposed to 2-chloracetamide/kg dry soil.

2. Observations:

Mortality: Dead earthworms were counted after 7 and 14 days.

Behaviour and appearance: Abnormal effects were noted after 7 and 14 days.

Mean Body Weights: All surviving earthworms per replicate were weighed as a group and average individual weights were calculated prior to test initiation and at day 14 after application.

3. Statistical calculations: NOEC and LOEC were calculated using a non-parametric procedure (Steel's Many-one Rank Test, one-tailed) with the computer program Toxstat v 3.4.Biomass was compared to the control using a two-tailed t-test.

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II. RESULTS AND DISCUSSION

A. FINDINGS

The 14 d LC_{50}, NOEC and LOEC values are given below based on nominal concentrations.

Endpoints	Glyphosate acid (mg a.e./kg dry soil)
LC_{50} (14 d)	5600 – 10000
NOEC (14 d) mortality	5600
LOEC (14 d) mortality	10000
NOEC (14 d) weight loss	< 5600
LOEC (14 d) weight loss	5600

B. OBSERVATIONS

Table 8.9.1-1: Sublethal effects of glyphosate acid on earthworm

Test item (mg a.e./kg dry soil)	Average Weight (mg/test vessel)								
	I		II		III		IV		
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Mean % weight change
Control	0.361	0.328	0.391	0.360	0.366	0.331	0.345	0.319	-8.5
5600	0.336	0.290	0.384	0.324	0.368	0.325	0.390	0.329	-14.2*
10000	0.366	0.355	0.380	0.260	0.366	0.305	0.407	0.255	-22.1

* Significantly different when compared to the control, ($\alpha = 0.05$)

Table 8.9.1-2: Lethal effects of glyphosate acid on earthworm

Test item (mg a.e./kg dry soil)	Number of mortalities in vessels								
	I		II		III		IV		
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Mortality after 14 d [%]
Control	0	0	0	0	0	0	0	0	0
5600	1	1	0	0	0	0	0	0	2.5
10000	3	8	4	8	2	8	3	8	80*

* Significantly different when compared to the control, ($\alpha = 0.05$)

Mortality: Mortality was observed at both test concentrations. At 5600 mg a.e./kg dry soil 2.5% mortality and at 10000 mg a.e./kg dry soil 80%. No mortality was observed in the negative control.

Mean Body Weight: The average life weights of the earthworms decreased significantly at 10000 mg a.e./kg dry soil.

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Behaviour and appearance: Surviving worms were recorded as lethargic and of emaciated appearance at 10000 mg a.e./kg dry soil and day 7 and 14. Two worms showed convulsive movements on day 7. At 5600 mg ae./kg dry soil no abnormalities of behaviour or appearance were noted.

Reference item: The 14-day LC_{50} of 2-chloroacetamide/kg dry soil was 47.9 mg/ kg dry soil (95% CL: 43.8 – 51.9). This LC_{50} compares well with published data and is within the limit of 20 – 80 mg specified in ISO 11268-1 (1993).

The validity criteria according to OECD 207 are fulfilled as no mortality was observed in the control group (required: < 10%).

III. CONCLUSION

The 14-day LC_{50} for earthworms (*Eisenia fetida*) exposed to glyphosate acid in an artificial substrate was determined to be >5770 – 10300 mg a.s./kg dry soil (5660 – 10000 mg a.e./kg dry soil). The corresponding no-mortality concentration was 5770 mg a.s./kg dry soil (5600 mg a.e./kg dry soil). The corresponding NOEC was determined to be < 5770 mg a.s./kg dry soil (< 5600 mg a.e./kg dry soil) due to effects on biomass change.

Annex point	Author(s)	Year	Study title
IIA 8.9.1/02	[redacted]	2000	AMPA: Acute toxicity of AMPA technical material to the earthworm *Eisenia andrei* in an artificial soil test [redacted] Report No: F13[redacted] Date: 2000-09-13 GLP: yes Not published

Guideline: OECD 207

Deviations to OECD 207: None

Dates of experimental work: 2000-05-30 to 2000-07-05

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Executive Summary

In a laboratory study, adult earthworms (*Eisenia andrei*) were exposed in a limit test for 14 days to two test concentrations of AMPA in artificial soil containing 10% sphagnum peat and observed for mortality and growth. A negative control group was maintained concurrently. Four replicate test chambers for the test item and the control were maintained in each treatment with 10 worms in each test chamber. Nominal test concentrations were 100 and 1000 mg AMPA/kg dry soil. After 14 days, number and weight of surviving adult worms was determined.

No mortality, morphological or behavioural alterations were observed in any test item treatment and the control. A significant difference in biomass was observed in the 1000 mg AMPA/kg dry soil test item concentrations when compared to the control. The validity criteria according to guideline OECD 207 are fulfilled.

The LC_{50} of *Eisenia andrei* exposed to AMPA was determined to be > 1000 mg a.s./kg dry substrate. The NOEC value for biomass was determined to be 100 mg a.s./kg dry substrate.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	AMPA (aminomethyl phosphonic acid)
Description:	White powder
Lot/Batch #:	0693/07866
Purity:	100%
2. Vehicle and/or positive control:	Chloroacetamide
3. Test organism:	
Species:	Earthworm (*Eisenia andrei*)
Age:	synchronized adults with clitellum, approx.. 5 months
Weight:	444.4 ± 69.5 mg
Source:	[redacted]
Food:	none
Acclimation period:	At least 24 h
4. Environmental conditions:	
Temperature:	20-22°C
Photoperiod:	24 h light (400 – 800 lux)
pH:	5.7 – 6.4 (start) 5.7 – 6.2 (end)
Water content:	54.9 – 60.5% (start) 52.5 – 60.0% (end)

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: Clitellate adult earthworms were exposed to the test substance in an artificial soil substrate (OECD 207, 10% sphagnum peat, air dried, finely ground; 20% kaolin clay, approximately 69% industrial quartz sand and 0.3 – 1% g calcium carbonate). Four replicate test chambers were maintained in each treatment and for the controls, with 10 worms in each test chamber. Nominal test concentrations of 100 and 1000 mg AMPA/kg dry soil were thoroughly mixed into the soil substrate. The water content was adjusted to 50±10% dry weight using deionised water. Negative control soil was treated with deionised water only.

In a separate study, earthworms were exposed to the toxic reference substance chloroacetamide.

Temperature and light intensity were monitored continuously. Water content and pH were determined at the beginning and the end of the test.

2. Observations: Mortality and mean body weights: The earthworms were exposed to the test item for 2 weeks and counted and weighed per replicate at the beginning and after 7 and 14 days of exposure.

Behavioural changes: behavioural changes and morphological alterations were recorded after 7 and 14 days.

3. Statistical analysis: For biomass deviation, a one-way ANOVA followed by Dunnett's test was conducted (α = 0.05).

II. RESULTS AND DISCUSSION

A. FINDINGS

The LC_{50} and NOEC values are given below based on mean measured concentrations.

Endpoints	Test item [mg a.s./kg dry soil]
LC_{50} (14 d)	>1000
$NOEC_{mortality}$ (14 d)	1000
$LC_{50, biomass}$ (14 d)	>1000
$NOEC_{biomass}$ (14 d)	100

B. OBSERVATIONS

No mortality was observed in any test item treatment concentrations or the control. After 14 days of exposure, mean percent biomass deviation was -21.3% in the control group, -25.3% for the 100 mg AMPA/kg dry soil and -35.1% for the 1000 mg AMPA/kg dry soil treatments. The ANOVA and Dunnett-test showed a significant difference in biomass between control and the highest test item concentration (α = 0.05). No treatment related effects on behaviour or morphology of earthworms were observed.

Table 8.9.1-3: Effects of AMPA on survival and growth of *Eisenia andrei*

	Control	AMPA [mg/kg dry soil]	
		100	1000
Mortality (day 14) [%]	0	0	0
Biomass (day 14) [mg fresh weight][1)]	346.8	333.6	289.8
Weight change (day 14) [%][1)]	-21.3	-25.3	-35.1

[1] negative values indicate a decrease of weight

The LC_{50} for the reference test item was determined to be 24.0 mg a.s./kg dry soil.

The mortality in the control treatments did not exceed 10%. The validity criteria according to guideline OECD 207 are therefore fulfilled.

III. CONCLUSION

At the highest test concentration, no effects on mortality, behaviour or appearance of *Eisenia Andrei* were observed after 14 days. Therefore, the LC_{50} of AMPA was determined to be > 1000 mg AMPA/kg dry substrate. The NOEC value for biomass change was determined to be 100 mg AMPA/kg dry substrate.

IIA 8.9.2 Sublethal effects on earthworms (and effects on other soil non-target macro-organisms)

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Annex point	Author(s)	Year	Study title
IIA 8.9.1/01	[redacted]	2009	MON 0139 - Sublethal toxicity to the earthworm *Eisenia fetida* [redacted] [redacted] Germany Report No: 09 10 48 056 S Date: 2009-11-30 GLP: yes Not published

Guideline: OECD 222 (2004)

Deviations to OECD 222: None

Dates of experimental work: 2009-08-28 to 2009-10-23

Executive Summary

The effects of MON 0139 (glyphosate isopropylamine salt) on *Eisenia fetida andrei* were tested in a 56 day sublethal laboratory test with regard to the parameters mortality, behavioural and pathological symptoms, body weight change and reproduction in OECD soil containing 10% peat moss. The test was conducted with five nominal test concentrations, encompassing 30, 50, 100, 500 and 1000 mg MON 0139/kg dry soil, equivalent to analysed content of 14.1, 23.6, 47.3, 236, and 473 mg glyphosate acid equivalent/kg dry soil respectively. In addition a control group was exposed to soil mixed with deionised water only.

After 56 days, the test item caused no mortality at the tested concentrations of 30, 500 and 1000 mg MON 0139/kg dry soil. 2.5 % mortality was observed at 50 and 100 mg MON 0139/kg dry soil. The mortality results did not reflect a dose related effect. No mortality occurred in the control group. No effects on behaviour (including feeding activity) of the worms were observed during the test. The test item caused no statistically significant change in biomass when compared to the control group. All validity criteria according to the OECD guideline 222 were fulfilled.

The EC_{50} of MON 0139 for earthworm was determined to be > 1000 mg test item/kg dry soil, equivalent to > 473 mg glyphosate acid equivalent/kg dry soil. The overall no observed effect concentration (NOEC) was determined to be 1000 mg/kg dry soil equivalent to 473 mg glyphosate acid equivalent/kg dry soil.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 0139 (glyphosate isopropylamine salt)
Description:	Pale yellow liquid
Lot/Batch #:	A8B60170S0
Purity:	63.81% w/w glyphosate isopropylamine salt (analysed) 47.28% w/w glyphosate acid equivalent (analysed)
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	Earthworm (*Eisenia fetida andrei*)
Age:	Adults, approx. 3 months old with clitellum
Weight:	304 – 472 mg
Source:	[redacted]
Food:	Air-dried and finely ground horse manure
Acclimation period:	Approx. 24 hours in the artificial substrate
4. Environmental conditions:	
Temperature:	18.6 – 21.8 ºC
Photoperiod:	16 h light / 8 h dark (600 Lux)
Soil pH:	6.1 – 6.2 (test start); 6.0 – 6.1 (test termination)
Soil moisture content:	35.1 – 35.2 % (test start); 34.6 – 34.8 % (test termination)

B. STUDY DESIGN AND METHODS

1. Experimental treatments: A sublethal test was conducted with five nominal test concentrations of MON 0139, encompassing 30, 50, 100, 500 and 1000 mg test item/kg dry soil, equivalent to an analysed content of 14.1, 23.6, 47.3, 236, and 473 mg glyphosate acid equivalent/kg dry soil, respectively. In addition, a control group was exposed to soil mixed with deionised water only. The test concentrations were prepared by dispersing an exactly weighed amount of the test item in deionised water (stock solutions) and thereafter diluted to obtain different test concentrations, which were thoroughly mixed with the artificial soil, achieving desired test doses with a final nominal water content of 40-60 % of WHC. The artificial soil substrate was composed of 10% Sphagnum-peat; 20% kaolin clay, 69.5% industrial quartz sand and 0.5% calcium carbonate. Four replicate test containers (test item) and 8 replicate test containers (control) with 810 g soil wet weight (corresponding to 600 g dry weight) and 5 cm soil depth were prepared for each treatment group. 10 adult earthworms per replicate (a total of 40 worms) were exposed for 56 days.

2. Observations: At test initiation, individual fresh weight and behavioural responses of earthworms were recorded. Behavioural and pathological symptoms including feeding activity were observed on a weekly basis. Four weeks after test initiation, number of surviving adult earthworms and fresh weight of surviving adult earthworms per replicate were recorded. At test termination (8 weeks after test initiation), number of surviving juveniles per replicate, were observed.

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The behavioural and pathological symptoms, including morphological alterations were observed 4 and 8 weeks after test initiation. Water content and pH measurements were performed at test initiation and at test termination. The temperature was continuously recoded throughout the test.

3. Statistical calculations: Fisher's Exact Binomial Test and Dunnett's t-test were used for mean comparison. For statistical evaluation of the biomass change, mean fresh weight of surviving worms was used.

II. RESULTS AND DISCUSSION

A. FINDINGS

Table 8.9.2-1: Lethal and sublethal effects of MON 0139 (glyphosate isopropylamine salt) on earthworm

MON 0139 [mg test item/kg dry soil]		Control	30	50	100	500	1000
Mortality of adult worms after 4 weeks (%)		0	0	2.5	2.5	0	0
Mean biomass change (%)		40.7	46.7	39.8	41.8	37.5	36.3
Mean number of juveniles after 8 weeks		79.0	78.5	83.8	71.8	80.3	74.3
CV %		18.7	19.1	15.0	34.1	28.7	22.1
Change of reproduction compared to control (%)		-	0.6	-6.0	9.2	-1.6	6.0
EC_{50}	Test item (MON 0139)	> 1000 mg/kg dry soil					
	glyphosate isopropylamine salt	> 638.1/kg dry soil					
NOEC	Test item (MON 0139)	1000 mg/kg dry soil					
	glyphosate isopropylamine salt	638.1/kg dry soil					

B. OBSERVATIONS

The test item MON 0139 caused no mortality at concentrations of 30, 500 and 1000 mg MON 0139/kg dry soil. 2.5 % mortality was observed at 50 and 100 mg MON 0139/kg dry soil. No mortality (0 %) occurred in the control group. The mortality results did not reflect a dose related effect. No effects on behaviour (including feeding activity) of the worms were observed during the test. The test item caused no statistically significant change in biomass (change in fresh weight after 4 weeks relative to initial fresh weight) when compared to the control.

The validity criteria according to guideline OECD 222 are fulfilled as each replicate (containing 10 adults) has produced ≥ 30 juveniles by the end of the test in the control and the coefficient of variation of reproduction was ≤ 30 % in the control. Also, the adult mortality over the initial 4 weeks of the test was ≤ 10 % in the control.

III. CONCLUSION

The EC_{50} of MON 0139 for earthworm was determined to be > 1000 mg test item/kg dry soil, equivalent to > 473 mg glyphosate acid equivalent/kg dry soil. The overall NOEC was determined to be 1000 mg/kg dry soil, equivalent to 473 mg glyphosate acid equivalent/kg dry soil.

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Annex point	Author(s)	Year	Study title
IIA 8.9.2/02	[redacted]	2000	A laboratory investigation of the effects of glyphosate and its breakdown product AMPA on reproduction in the earthworm *Eisenia fetida*. [redacted] Report No: [redacted]-1173 Date: 2000-03-27 GLP: yes Not published

Guideline: ISO 11268-2 (1998)
OECD Guideline No. 222

Deviations to OECD 222: None

Dates of experimental work: 1999-10-20 to 1999-12-15

Executive Summary

The effects of the isopropylamine (IPA) salt of glyphosate and the metabolite aminomethylphosphonic acid (AMPA), on the earthworm *Eisenia fetida* were tested in a 56 days chronic laboratory test with regard to the parameters mortality, development of body weight and reproduction. The test was conducted with two test concentrations of glyphosate IPA salt (5.76 and 28.79 mg/kg dry soil (equivalent to 4.27 and 21.31 mg glyphosate acid equivalent/kg dry soil)) and two test concentrations of AMPA (5.62 and 28.12 mg/kg dry soil) in OECD soil containing 10% peat moss. Furthermore, a negative and three concentrations of a positive control ([redacted]) were tested.

Only one adult worm died during the test at the lowest concentration of glyphosate IPA salt (5.76 mg/kg dry soil) tested and thus was not considered to be dose-related. Furthermore, no significant difference in body weight change compared to the untreated controls was noted for adult worms exposed to the glyphosate IPA salt or AMPA at any of the concentrations tested in this study.

No significant differences were observed between the mean juvenile production for the untreated control worms and specimens exposed to glyphosate IPA or AMPA at any concentration tested. Similarly, no significant differences were observed between the numbers of unhatched cocoons present at day 56 in the untreated controls and those in both concentrations of glyphosate IPA salt or AMPA. All validity criteria according to the OECD guideline 222 were fulfilled.

In conclusion, glyphosate, tested as glyphosate IPA salt, and the metabolite aminomethylphosphonic acid (AMPA), had no significant effects on growth or reproduction of *Eisenia fetida* at concentrations up to 28.79 mg glyphosate IPA salt/kg dry soil (21.3 1 mg glyphosate acid equivalent/kg dry soil) and 28.12 mg AMPA/kg dry soil. Therefore, the NOEC were determined to be 28.79 mg glyphosate IPA salt/kg dry soil (21.31 mg glyphosate acid equivalent/kg dry soil) and 28.12 mg AMPA/kg dry soil.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item 1: Isopropylamine (IPA) salt of glyphosate
Description: Clear liquid
Lot/Batch #: A9C 281
Purity: 62% (45.9% glyphosate acid equivalent)

Test item 2: AMPA (aminomethylphosphonic acid)
Description: White crystalline powder
Lot/Batch #: PIT-8912-1385-A
Purity: 99.1%

2. Vehicle and/or positive control:

Positive control: [redacted] (50% w/w benomyl)
Reference item (in a separate study): 2-chloroacetamide

3. Test organism:

Species: Earthworm (*Eisenia fetida*)
Age: Adults, 7-10 month old
Weight: 386 – 477 mg (test initiation)
Source: [redacted] UK
Food: Cattle manure
Acclimation period: Earthworms were acclimatised to the artificial soil for a period of 29 days at 16 – 22.5°C.

4. Environmental conditions:

Temperature: 18 - 22°C
Photoperiod: 16 h light: 8 h dark
Soil pH: 6.38 – 6.60 (test initiation); 6.83 - 6.96 (test termination)
Soil temperature: 18.4 – 19.6°C
Soil moisture content: 37.9% (60 % of the water holding capacity) (test initiation); 29.6 – 31.1% (test termination)

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: The test was conducted with two test concentrations of glyphosate IPA salt (5.76 and 28.79 mg/kg dry soil, equivalent to 4.27 and 21.31 mg glyphosate acid equivalent/kg dry soil), two test concentrations of AMPA (5.62 and 28.12 mg/kg dry soil), a negative control, and three concentrations of a positive control ([redacted]).The test item was suspended into/dissolved in deionised water and the suspension/solution was mixed with the water used for adjusting the soil moisture to 60% of the water holding capacity. Afterwards, the solution was mixed into the artificial soil substrate (10% peat; 20% clay, 70% silica sand and calcium carbonate to obtain a pH of 5.5-6.5). 1 g cow manure/ 100 g dry soil was added as feed. Four replicate test containers with 600 g dry soil were prepared for each treatment group. 10 adult earthworms per replicate (a total of 40 worms) were exposed for 56 days. Earthworms

were fed with manure on day 1, 14, 21 and 28. Soil moisture was adjusted once a week by adding deionised water. A negative control was treated with deionised water only. As positive control, earthworms were exposed to three concentrations of (2.66, 5.93 and 13.28 mg/kg).Temperature and light intensity were recorded daily during the test period. pH and soil temperature were determined at the beginning and the end of the test in one of the replicate vessels at each concentration. Soil moisture content was determined at day 0, 1, 7, 14, 21, 23, 28, 35, 42 and 56. Furthermore, toxicity of 2-chloroacetamide to *Eisenia fetida* was tested in a separate 14 d reference study.

2. Observations:

Mortality and reproduction: The replicates were examined for live and dead adult worms after 28 days at which time all adult worms were removed and the soil was replaced in the vessels. After a further 28 days (56 days total), the contents of the beakers were examined for juvenile worms and cocoons.

Mean body weights: All surviving earthworms per replicate were weighed as a group and average individual weights were calculated prior to test initiation and at day 28 after application.

3. Statistical calculations: Mean percent changes in weights of live worms at 28 days and mean juvenile production per surviving adult worm at day 56 were tested for significant ($\alpha = 0.05$) inhibition compared to the controls using the Dunnett's Test (one tailed comparison) in the computer program TOXSTAT Release 3.0. The same test, but with a two-tailed comparison, was employed to test for significant differences between mean numbers of un-hatched cocoons because the test substances may have inhibited cocoon production or/and cocoon viability (cocoons may have been produced but unable to hatch). Each set of data was tested for normality before carrying out the parametric multiple comparison procedure using the Chi-square test and the Shapiro Wilks test, the data were also tested for homogeneity of variance using both the Hartley and the Bartlett's tests provided in the program TOXSTAT Release 3.0.

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II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

Table 8.9.2-2: Summary of the effects of glyphosate IPA salt, AMPA and the positive control ███ on *Eisenia fetida*

Treatment	Adult worms		Juvenile production (at day 56)		Mean number of unhatched cocoons per surviving worm
	Percentage mortality of adult worms (at day 28)	Mean percent weight change (at day 28)	Mean number of juveniles per surviving worm	Coefficient of variation	
Control	0	+ 22	31.0	10	0.1
███ (2.66 mg/kg)	0	+ 23	26.0 *	15	0.1
███ (5.93 mg/kg)	0	+ 12 *	7.8 *	23	2.2 *
███ (13.28 mg/kg)	0	- 24 *	0.0 *	0	0.7
Glyphosate[1] (5.76 mg IPA salt/kg)	2.5	+ 14	26.2	25	0.3[(N)]
Glyphosate[1] (28.79 mg IPA salt/kg)	0	+ 20	28.5	12	0.3[(N)]
AMPA (5.62 mg/kg)	0	+ 24	26.0	3	0.3[(N)]
AMPA (28.12 mg/kg)	0	+ 24	29.4	16	0.4[(N)]

* statistically (α = 0.05) different from controls.
1 glyphosate was tested as the IPA salt.
N The numbers of unhatched cocoons present at the end of the test in the glyphosate and AMPA treatments were slightly higher than the controls but statistical analysis proved that this was probably due to random chance alone and was probably not due to the presence of glyphosate or AMPA

B. OBSERVATIONS

Mortality: Only one adult worm died during the test at the lowest concentration of glyphosate IPA salt (5.76 mg/kg dry soil). This was not considered to be dose-related since no mortalities were observed at higher concentrations.

Mean body weight: No significant difference in body weight change compared to untreated controls was noted for adult worms at any concentration or test item treatment.

Behaviour: No abnormal behaviour when compared to untreated controls was observed for adult worms at any concentration or test item treatment.

Reproduction: No significant differences were observed between mean juvenile production for untreated control worms and worms exposed to glyphosate IPA at any concentration tested. Similarly, for worms exposed to AMPA no significant difference from the untreated controls was seen in terms of juvenile production. No significant differences were observed between number of unhatched cocoons present at

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day 56 in untreated controls and both concentrations of glyphosate IPA salt. Similarly, for AMPA, no significant difference from the controls was observed in terms of numbers of unhatched cocoons.

Positive control: The adult worms exposed to 5.93 and 13.28 mg [redacted]/kg dry soil showed a significantly reduced growth when compared to untreated controls at day 28. A significant reduction in juvenile production compared to untreated controls was seen for 2.66, 5.93 and 13.28 mg [redacted]/kg dry soil. At 5.93 mg [redacted]/kg dry soil a significantly increased number of unhatched cocoons was observed when compared to the untreated control.

Reference study with 2-chloroacetamide: The 14 day LC_{50} was determined at 39.4 mg/kg dry soil (95% confidence limits; 36.0 - 43.1 mg/kg dry soil).

The validity criteria according to guideline OECD 222 are fulfilled as each replicate (containing 10 adults) have produced ≥ 30 juveniles by the end of the test in the control and the coefficient of variation of reproduction was ≤ 30 % in the control. Also, the adult mortality over the initial 4 weeks of the test was ≤ 10 % in the control.

III. CONCLUSION

In conclusion, glyphosate, tested as glyphosate IPA salt, and the metabolite aminomethylphosphonic acid (AMPA) had no significant effect on growth or reproduction of *Eisenia fetida* after 56 days of exposure at concentrations up to 28.79 mg glyphosate IPA salt/kg dry soil (21.3 1 mg glyphosate acid equivalent/kg dry soil) and 28.12 mg AMPA/kg dry soil. Therefore, the NOEC was determined to be 28.79 mg glyphosate IPA salt/kg dry soil (21.3 1 mg glyphosate acid equivalent/kg dry soil) and 28.12 mg AMPA/kg dry soil.

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Annex point	Author(s)	Year	Study title
IIA 8.9.2/03	[redacted]	2002	AMPA Earthworm (*Eisenia fetida*), Effects on Reproduction [redacted] Report No: 011120[redacted] Date: 2002-06-05 GLP: yes Not published

Guideline: Soil quality – effects of pollutants on earthworms (*Eisenia fetida*, *Eisenia fetida andrei*) – Determination of effects on reproduction DIN ISO 11268-2: 1998E

Deviations to OECD 222: None

Dates of experimental work: 2002-01-31 to 2002-03-28

Executive Summary

In a laboratory study, adult earthworms (*Eisenia fetida*) were exposed for 28 days to three test concentrations of AMPA in artificial soil containing 10% sphagnum peat and observed for mortality, growth, and reproduction. A negative control group was maintained concurrently. Four replicate test chambers were maintained in each treatment with 10 worms in each test chamber. Nominal test concentrations were 0.79, 3.94 and 19.7 mg AMPA/kg dry soil. After 28 days, number and weight of surviving adult worms was determined. After a further 28 days the reproduction rate was determined by counting the numbers of juvenile earthworms and cocoons in each test vessel.

No mortality was observed in any treatment group. The body weight of the earthworms exposed to AMPA were not statistically different when compared to the control up to and including the highest test concentration of 19.7 mg AMPA/kg dry soil. Moreover, no statistically significant effects on the reproduction were observed up to and including the highest test concentration of 19.7 mg AMPA/kg dry soil. No behavioural abnormalities were observed in any of the treatment groups. The validity criteria according to the current guideline OECD 222 are fulfilled.

The56-day no-observed-effect-concentration (NOEC)of AMPA for mortality, body weight and the reproduction rate of *Eisenia fetida* was 19.7 mg AMPA/kg dry soil, which was the highest concentration tested.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: AMPA (aminomethyl phosphonic acid)
Description: White powder
Lot/Batch #: FA005563
Purity: 99%

2. Vehicle and/or positive control: ████ (31.5% carbendazim)

3. Test organism:

Species: Earthworm (*Eisenia fetida*)
Age: synchronized adults with clitellum, 4 months
Weight: 300 – 600 mg
Source: ████ Germany
Food: Dried litter of stinging nettle and porridge oats
Acclimation period: 2 days in artificial soil under test conditions

4. Environmental conditions:

Temperature: 20 ± 2°C
Photoperiod: 16 h light/ 8 hours dark (413 - 546 lux)
pH: 5.45 – 6.30
Water content: 46.11 – 51.53%

B: STUDY DESIGN AND METHODS

1. Experimental treatments: Clitellate adult earthworms were exposed to the test substance in an artificial soil substrate (OECD 207; 10% sphagnum peat, air dried, finely ground; 20% kaolin clay, approximately 69% industrial quartz sand and approx. 0.43% calcium carbonate).Four replicate test chambers were maintained in each treatment, with 10 worms in each test chamber. Nominal test concentrations of0.79, 3.94 and 19.7 mg AMPA/kg dry soil were thoroughly mixed into the soil substrate. The water content was adjusted to about 50% of maximum water holding capacity (WHC) using demineralised water. Negative control soil was treated with deionised water only.

As a toxic reference, earthworms were exposed in a separate study to ████ (31.5% carbendazim) at concentrations of 1.26, 2.52 and 5.04 mg a.s./kg dry soil.

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The adult earthworms were exposed to the test item for 4 weeks; the adult worms were counted, removed and weighed per replicate. The remaining soil was returned to the reproductive test for additional 4 weeks. Thereafter, juveniles were counted. Temperature and light intensity were monitored continuously. Water content and pH were determined at the beginning and the end of the test.

2. Observations: Mortality: The adult earthworms were exposed to the test item for 4 weeks, after which the artificial soil was emptied onto a tray and the adult worms were counted, removed and weighed per replicate after they were washed under tap water and dried on filter paper. Missing worms and the earthworms, which failed to respond to gentle stimulation, were considered to be dead.

Behavioural abnormalities: The number of damaged earthworms (e.g. lack of movement, rigidity, etc.) was assessed at day 28 after application.

Mean Body Weights: Individual weight of the earthworms was recorded at day 28 after application.

Reproduction: Reproduction was recorded8 weeks after the test initiation as mean number of juveniles per test container and replicate.

3. Statistical analysis: As data for body weight changes and the reproduction were normally distributed and homogeneous, the Dunnett's test was used (multiple comparison, two-sided for weight and one sided smaller for reproduction, $\alpha = 0.05$). NOEC and EC_x-values for reproduction were determined by regression analysis in an appropriate dose-response function.

II. RESULTS AND DISCUSSION

A. FINDINGS

The LC_{50}, EC_{50} and NOEC values are given below based on nominal concentrations.

Endpoints	AMPA [mg/kg dry soil]
LC_{50} (28 d)	>19.7
$NOEC_{mortality}$ (28 d)	19.7
$EC_{50, biomass}$ (28 d)	>19.7
$NOEC_{biomass}$ (28 d)	19.7
$EC_{50, repro}$ (56 d)	>19.7
$NOEC_{repro}$ (56 d)	19.7

B. OBSERVATIONS

No pathological symptoms or chances in behaviour of the adult earthworms were noted in any of the test item treatments and the control. During test period, body weights of earthworms in treated and control groups slightly increased or remained at starting level. No mortality was observed in any of the treatment groups and in the control. Different test item concentrations had no effects on the number of offspring. There was no statistically significant difference between the treated groups and the control.

Table 8.9.2-3: Effects of AMPA on survival, growth and reproduction of *Eisenia fetida*

	Control	AMPA [mg/kg dry soil]		
		0.79	3.94	19.7
Mortality (day 28) [%]	0	0	0	0

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Weight change (day 28) [%][1]	-	+10.71	+1.79	+7.14
No of juveniles (day 56)	60±23	64±23	61±5	68±10
Reproduction [%] of control (56 days)[1)]	-	+7	+2	+13

1 negative values indicate a decrease, positive values an increase when compared to the control

The $EC_{50}$and NOEC for reproduction in the reference test item were determined to be 2.9 and 1.26 mg a.s./kg dry substrate, respectively.

Each control replicate containing 10 adults produced ≥ 30 juveniles and adult mortality in the control treatments after four weeks did not exceed 10%. The coefficient of variation for reproduction in control groups was slightly higher than 30% at the end of the test. As one-way ANOVA showed that replicates were normally distributed and homogeneously with regard to variance, the validity criteria according to guideline OECD 222 are therefore still considered fulfilled.

III. CONCLUSION

The 56-day no-observed-effect-concentration (NOEC) of AMPA for mortality, growth and reproduction of the earthworm *Eisenia fetida* was found to be 19.7 mg AMPA/kg dry soil, which was the highest concentration tested.

Annex point	Author(s)	Year	Study title
IIA 8.9.2/04		2003	Laboratory determination of the side-effects of aminomethyl phosphonic acid (AMPA) on the reproductive performance of earthworms (*Eisenia fetida*) using artificial soil substrate Report No: 01-64-077- Date: 2003-01-20 GLP: yes Not published

Guideline: OECD 222 (draft, January 2000)

Deviations to OECD 222: None

Dates of experimental work: 2002-05-07 to 2003-01-08

Executive Summary

In a laboratory study, adult earthworms (*Eisenia fetida*) were exposed for 28 days to eight test concentrations of AMPA in artificial soil containing 10% sphagnum peat and observed for mortality, growth, and reproduction. A negative control group was maintained concurrently. Four replicate test chambers for the test item and eight test chambers for the control were maintained in each treatment with 10 worms in each test chamber. Nominal test concentrations were 58.6, 87.8, 131.9, 198.1, 297.1, 445.5, 668.5 and 1002.5 mg AMPA/kg dry soil. After 28 days, number and weight of surviving adult worms was determined. After a further 28 days the reproduction rate was determined by counting the numbers of juvenile earthworms in each test vessel.

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One earthworm died in one of the 668.5 mg AMPA/kg dry soil and in the 2.2 and 5.0 mg Carbendazim/kg dry soil test units each. Mean percent biomass deviation was -9.5% in the control group, ranging between -4.0 and -12.9%. Loss of biomass was of the same level in the AMPA treated groups for treatment concentrations of up to 297.1 mg AMPA/kg dry soil, except for the 198.1 mg AMPA/kg dry soil treatment group, where loss of biomass appeared to be significantly higher when compared to the control. F-variance analysis proved that the loss of biomass was significantly higher for treatment concentrations of 445.5 mg AMPA/kg dry soil and higher. A significant reduction of offspring was observed in AMPA treatment groups for treatment concentrations equal and higher than 297.1 mg/kg dry soil. The validity criteria according to the current guideline OECD 222 are fulfilled.

The 56-day no-observed-effect-concentration (NOEC) of AMPA for mortality, body weight and the reproduction rate of *Eisenia fetida* was 198.1 mg AMPA/kg dry soil, observed on earthworm reproduction.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	AMPA (aminomethyl phosphonic acid)
Description:	White powder
Lot/Batch #:	A0164351
Purity:	99.7%

2. Vehicle and/or positive control: Carbendazim (99.6%)

3. Test organism:

Species:	Earthworm (*Eisenia fetida*)
Age:	synchronized adults with clitellum, >2 months
Weight:	300 – 600 mg
Source:	[redacted]
Food:	5 g of cattle manure with 6 mL of water per week
Acclimation period:	Not stated

4. Environmental conditions:

Temperature:	19.0 to 21.5°C
Photoperiod:	12 h light / 12 hours dark (416 - 595 lux)
pH:	5.7 – 6.0 (start) 6.3 – 6.9 (end)
Water content:	43.9 – 46.2%

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: Clitellate adult earthworms were exposed to AMPA in an artificial soil substrate (OECD 207, 10% sphagnum peat, air dried, finely ground; 20% kaolin clay, approximately 68% industrial quartz sand and approx. 1.8 g calcium carbonate). Four replicate test chambers were maintained in each treatment and eight for the controls, with 10 worms in each test chamber. Nominal test concentrations 58.6, 87.8, 131.9, 198.1, 297.1, 445.5, 668.5 and 1002.5 mg AMPA/kg dry soil were thoroughly mixed into the soil substrate. The water content was adjusted to about 50% of maximum water holding capacity (WHC) using demineralised water. Negative control soil was treated with deionised water only.

As a toxic reference, earthworms were exposed to carbendazim at concentrations of 1.0, 2.2 and 5.0 mg a.s./kg dry soil.

The adult earthworms were exposed to the test item for 4 weeks; the adult worms were counted, removed and weighed per replicate. The remaining soil was returned to the reproductive test for additional 4 weeks. Thereafter, juveniles were counted. Temperature and light intensity were monitored continuously. Water content and pH were determined at the beginning and the end of the test.

2. Observations:

Mortality: The adult earthworms were exposed to the test item for 4 weeks, after which the adult worms were counted, removed and weighed per replicate after they were washed under tap water and dried. Missing worms and the earthworms, which failed to respond to gentle stimulation, were considered to be dead.

Mean Body Weights: Individual weight of the earthworms was recorded at day 28 after application and calculated as total weight reported to the number of survivals.

Reproduction: Reproduction was recorded 8 weeks after the test initiation as mean number of juveniles per test container and replicate.

3. Statistical analysis: For biomass deviation and production of juveniles, F-variance analysis was conducted ($\alpha = 0.01$). The EC-value for reproduction was determined by linear regression analysis.

II. RESULTS AND DISCUSSION

A. FINDINGS

The LC_{50}, EC_{50} and NOEC values are given below based on mean measured concentrations.

Endpoints	AMPA [mg/kg dry soil]
LC_{50} (28 d)	>1002.5
$NOEC_{mortality}$ (28 d)	1002.5
$LOEC_{, biomass}$ (28 d)	445.5
$NOEC_{biomass}$ (28 d)	297.1
EC_{50}, repro (56 d)	297.1 (381.2 – 774.1)
$LOEC_{, repro}$ (56 d)	297.1
$NOEC_{repro}$ (56 d)	198.1

B. OBSERVATIONS

Mean percent biomass deviation was -9.5% in the control group, ranging between -4.0 and -12.9%. Loss of biomass was of the same level in the AMPA treated groups for treatment concentrations of up to 297.1 mg AMPA/kg dry soil, except for the 198.1 mg AMPA/kg dry soil treatment group, where loss of biomass appeared to be significantly higher when compared to the control. F-variance analysis proved that the loss of biomass was significantly higher for treatment concentrations of 445.5 mg AMPA/kg dry soil and higher. A significant reduction of offspring was observed in AMPA treatment groups for treatment concentrations equal and higher than 297.1 mg/kg dry soil. One earthworm each died in one of the 668.5 mg AMPA/kg dry soil and in the 2.2 and 5.0 mg Carbendazim/kg dry soil test units.

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Table 8.9.2-4: Effects of AMPA on survival, growth and reproduction of *Eisenia fetida*

	Control	AMPA [mg/kg dry soil]							
		58.6	87.8	131.9	198.1	297.1	445.5	668.5	1002.5
Mortality (day 28) [%]	0	0	0	0	0	0	0	2.5	0
Weight change (day 28) [%][1)]	-9.5	-11.0	-10.0	-11.5	-16.8	-11.8	-22.3	-32.4	-34.2
No of juveniles (day 56)	120.6 ±12.4	114.8 ±12.1	112.5 ±9.8	110.0 ±14.8	109.0 ±11.2	93.8 ±10.2	66.8 ±3.4	41.0 ±3.2	16.3 ±6.3
Reproduction [%] of control (56 days)	-	n.s.	n.s.	-8.8	-9.6	-22.3	-44.7	-66.0	86.5

[1] negative values indicate a decrease of weight
n.s. not stated

The NOEC for reproduction in the reference test item was determined to be 2.2 mg a.s./kg dry soil, respectively.

Each control replicate containing 10 adults produced ≥ 30 juveniles and adult mortality in the control treatments after four weeks did not exceed 10%. The coefficient of variation for reproduction in control groups 10.3% at the end of the test. Therefore, all validity criteria according to guideline OECD 222 are fulfilled.

III. CONCLUSION

The 56-day no-observed-effect-concentration (NOEC) of AMPA for mortality, body weight and the reproduction rate of *Eisenia fetida* was 198.1 mg AMPA/kg dry soil, observed on earthworm reproduction.

Annex point	Author(s)	Year	Study title
IIA 8.9.2/05	[redacted]	2009	MON 0139 – Effects on the reproduction of the predatory mite *Hypoaspis aculeifer* [redacted] Report No: 09 10 48 058 S Date: 2009-12-18 GLP: yes not published

Guideline: OECD 226 (2008)

Deviations to OECD 226: None

Dates of experimental work: 2009-09-08 to 2009-10-06

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Executive Summary

In the laboratory study the toxicity and reproductive inhibition of MON 0139 (glyphosate isopropylamine salt) to *Hypoaspis aculeifer* was tested. Adult mites were exposed to 50, 100, 500 and 1000 mg MON 0139/kg dry soil (23.64, 47.28, 236.40 and 472.80 mg glyphosate acid equivalent/kg dry soil). In addition a blank control with deionised water and a toxic reference (███████) were tested.

40 mites (10/ test vessel) per test concentration and 80 mites per control (10/ test vessel) were put in a glass bottle on artificial soil with incorporated test item. Adults and juveniles were counted after 14 d. The test item MON 0139 caused no statistically significant mortality of adult *Hypoaspis aculeifer* at the end of the 14-day exposure period. Also no significant decrease in reproduction was observed. All validity criteria according to OECD 226 were fulfilled.

In conclusion, in a 14 d laboratory test to determine the effects of MON 0139 on the predatory mite, *Hypoaspis aculeifer*, the 14-d EC_{50} was >1000 mg MON 0139/ kg dry soil (472.80 mg glyphosate acid equivalent/kg dry soil). The NOEC was 1000 mg test item/kg dry soil (472.80 mg glyphosate acid equivalent/kg dry soil), the highest tested concentration, since MON 0139 had no negative effect on the test organisms.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 0139 (glyphosate isopropylamine salt)
Description:	Pale yellow liquid
Lot/Batch #:	A8B60170S0
Purity:	Nominal: 62 % w/w glyphosate isopropylamine salt(corresponding to 45.9% w/w glyphosate acid equivalent) Analysed: 63.81 ± 0.29% w/w glyphosate isopropylamine salt (corresponding to 47.28 ± 0.21% w/w glyphosate acid equivalent)

2. Vehicle and/or positive control: Reference item: ███████ (Dimethoate, EC 400, 422.4 g/L analysed)

3. Test organisms:

Species:	*Hypoaspis aculeifer* (Canestrini)
Age:	Adult mites
Source:	███████████████████ ███████ Germany
Diet/Food:	*Tyrophagus putrescentiae* (Schrank) were fed every 2 days, before and during the test

4. Environmental conditions:

Temperature:	19.7 – 21.9 °C
Composition of artificial soil	5% sphagnum peat 20% kaolin clay 0.3% calcium carbonate 74.7% quartz sand Deionised water
Soil water content:	Test start: 18.79 – 20.21% (47.52 – 51.11 % of WHC) Test end: 18.65 – 20.11% (47.17 – 50.87% of WHC)

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pH	Test start: 5.9 – 6.2 Test end: 5.3 – 5.4
Photoperiod:	16 hours light / 8 hours darkness
Light intensity:	588 lux

B: STUDY DESIGN AND METHODS

1. Experimental treatments: MON 0139 was evaluated for mortality and reproductive reduction in a test with *Hypoaspis aculeifer* at four application rates, equivalent to 50, 100, 500 and 1000 mg MON 0139/kg dry soil (= 23.64, 47.28, 236.40 and 472.80 mg glyphosate acid equivalent/kg dry soil). In addition a blank control with deionised water and a toxic reference (422.4 g/L dimethoate) were tested.

Each test item concentration was tested with 40 mites (10/ test vessel), while the control group consisted of 8 replicates. For each test item concentration and for the control group 2 test vessels without mites were provided for measurement purposes.

The mites were put in glass bottles with screw tops of 100 mL containing 20 g (dry weight) artificial soil with the requested test item concentrations and closed, but opened every second day for food supply and aeration. Two weeks after introducing the test organisms the parental and juvenile were counted.

2. Observations: Water content and pH were determined at test start and end. Adult and juvenile mites were counted at test end.

3. Statistical calculations: The statistical analysis was performed with the software ToxRat Professional 2,10. Fisher's Exact Binomial Test with Bonferroni Correction and Dunnett's test (α = 0.05) were used to compare the control with independent test item groups. Abbott's formula was used to correct for control mortality.

II. RESULTS AND DISCUSSION

A. FINDINGS

The EC_{50} value and the NOEC are given below based on nominal concentrations.

Endpoints	MON 0139 [mg/kg dry soil]	Glyphosate acid equivalent [mg/kg dry soil]
NOEC	1000	472.80
EC_{50} (14 d)	> 1000	>472.80

Reference test:

After treatment with the reference item (Dimethoate, EC 400, 422.4 g/L analysed) at concentrations of 4.1, 5.12, 6.40, 8.00 and 10.00 mg a.s./ kg dry soil an EC_{50} (reproduction) of 4.9 mg test item/ kg dry soil was calculated.

B. OBSERVATIONS

The test item MON 0139 caused no statistically significant mortality (Fishers' Exact Binominal Test, α = 0.05) of the adult *Hypoaspis aculeifer* at the end of the 14-day exposure period. Also no significant decrease in reproduction was observed (Dunnett-t-test, α = 0.05).

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Table 8.9-5: Mortality and reproductive reduction of *Hypoaspis aculeifer* after application of AMPA in a 14 d laboratory study

Test rate [MON 0139/kg dry soil]	Test rate [glyphosate acid equivalent/kg dry soil]	Mortality of parental collembolans after 14days [%]	Corrected mortality [1] [%]	Mean number of juveniles after 14 days [%]	Reduction of reproduction compared to control [%]
Control	Control	8.8	-	190.5	-
50	23.64	10	1.4	176.8	7.2
100	47.28	12.5	4.1	173.5	8.9
500	236.40	1.0	1.4	182.3	4.3
1000	472.80	7.5	-1.4	207.8	-9.1

[1] calculated with Abbott 1925

Reference test:

Treatment with the reference item [redacted] at a concentration of 4.1, 5.12, 6.40, 8.00 and 10.00 mg a.s./ kg dry soil resulted in significant effects on reproduction.

All validity criteria according to OECD 226 were fulfilled, as adult mortality in the control treatments did not exceed 20%, the mean number of juveniles per replicates was ≥ 50 at test end and the coefficient of variation of the number of juveniles per replicate was not higher than 30% at test end.

III. CONCLUSION

In a 14 d laboratory test to determine the effects of MON 0139 on the predatory mite, *Hypoaspis aculeifer*, the 14-d EC_{50} was >1000 mg MON 0139/ kg dry soil (472.80 mg glyphosate acid equivalent/kg dry soil). The NOEC was 1000 mg test item/kg dry soil (=472.80 mg glyphosate acid equivalent/kg dry soil), the highest tested concentration, since MON 0139 had no negative effect on the test organisms.

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Annex point	Author(s)	Year	Study title
IIA 8.9.2/06		2010	MON 0139 – Effects on the reproduction of the collembolans *Folsomia candida* Report No: 09 10 48 057 S Date: 2010-02-02 GLP: yes not published

Guideline: OECD 232 (2009)
ISO 11267 (1999)

Deviations to OECD 232: None

Dates of experimental work: 2009-09-04 to 2009-10-02

Executive Summary

In a laboratory study the toxicity and reproductive inhibition of MON 0139 (Glyphosate isopropylamine salt) to *Folsomia candida* was tested. Juvenile springtails, approximately 10 - 12 days old, were exposed to 32, 50, 100, 500 and 1000 µL MON 0139/kg dry soil (= 19, 29, 59, 294 and 587 mg glyphosate acid equivalent/kg dry soil). In addition a blank control with deionised water and a toxic reference ( ) were tested.

50 springtails (10/ test vessel) per test concentration and control were put in a glass container on artificial soil with incorporated test item and adults and juveniles counted after 28 d. All validity criteria according to OECD 232 were fulfilled.

In conclusion, in a 28 d laboratory test to determine the effects of MON 0139 on *Folsomia candida*, the 28-d EC_{50} was > 1000 µL MON 0139/kg dry soil (587 mg glyphosate acid equivalent/kg dry soil). The NOEC was 1000 µg/L MON 0139/kg dry soil (587 mg glyphosate acid equivalent/kg dry soil), the highest tested concentration, since MON 0139 salt had no negative effect on the test organisms.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: MON 0139 (Glyphosate isopropylamine salt)
Description: Pale yellow liquid
Lot/Batch #: A8B60170S0
Purity: Nominal: 62 % w/w glyphosate isopropylamine salt (corresponding to 45.9% w/w glyphosate acid equivalent)
Analysed: 63.81 ± 0.29% w/w glyphosate isopropylamine salt (corresponding to 47.28 ± 0.21% w/w glyphosate acid equivalent)

2. Vehicle and/or positive control: Reference item: (Phenmedipham EC 157 g/L)

3. Test organisms:

Species:	*Folsomia candida* (Willem)
Age:	Juvenile springtails (10 – 12 d old)
Source:	[redacted] Germany
Diet/Food:	Approximately 2 mg granulated dry yeast at test start and after 14 days

4. Environmental conditions:

Temperature:	20.4 – 21.1 °C
Composition of artificial soil	10% sphagnum peat 20% kaolin clay 0.5% calcium carbonate 69.5% quartz sand Deionised water
Soil water content:	Test start: 34.9 – 35.2% (54.4 – 54.9 % of WHC) Test end: 34.5 – 34.7% (53.8 – 54.1% of WHC)
pH:	Test start: 6.01 – 6.08 Test end: 5.79 – 5.91
Photoperiod:	16 hours light / 8 hours darkness
Light intensity:	580 lux

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: MON 0139 was evaluated for mortality and reproductive reduction in a test with *Folsomia candida* at five application rates of 32, 50, 100, 500 and 1000 µLMON 0139/kg dry soil (19, 29, 59, 294 and 587 mg glyphosate acid equivalent/kg dry soil). In addition a blank control with deionised water and a toxic reference ([redacted]) were conducted. Each test item concentration and the control were tested with 50 springtails (10/ test vessel). For each test item concentration and for the control group 2 test vessels without springtails were provided for measurement purposes. The springtails were put in a glass container (~ 150 mL) containing 30g (wet weight) artificial soil with the requested test item concentrations and covered with a glass lid for 28 d. Four weeks after introducing the test organisms the surviving adults and juveniles were counted.

2. Observations: Water content and pH were determined at test start and end. Adults and juvenile springtails were counted at test end.

3. Statistical calculations: The statistical analysis was performed with the software ToxRat Professional 2.10. Fisher's Exact Binomial Test with Bonferroni Correction and Welch t-test were used to compare the control with the independent test item groups for significance of parental mortality and reproductive reduction, respectively. Abbott's formula was used to correct for control mortality.

II. RESULTS AND DISCUSSION

A. FINDINGS

The EC_{50} value and the NOEC for mortality and reproduction are given below based on nominal concentrations.

Endpoints	MON 0139 [µL/kg dry soil]	Glyphosate acid equivalent [mg/kg dry soil]
NOEC (mortality)	1000	587
NOEC (reproduction)	1000	587
EC_{50} (28 d)	> 1000	> 587

Reference test:

After treatment with the reference item ██████ (Phenmedipham EC 157 g/L) at concentrations of 50, 100, 200 and 400 mg test item/ kg dry soil an EC_{50} of 181.0 mg ██████/ kg dry soil was determined.

B. OBSERVATIONS

No statistically significant effects on parental mortality (Fishers's Exact Binominal Test, p > 0.05) or the number of offspring (Welch-t-test, α = 0.05) was found.

Table 8.9-6: Mortality and reproductive reduction of *Folsomia candida* after application of MON 0139 in a 28 d laboratory study

Test rate [µL MON 0139/kg dry soil]	Test rate [glyphosate acid equivalent/kg dry soil]	Mortality of parental collembolans after 4 weeks [%]	Corrected mortality [1] [%]	Mean number of juveniles after 4 weeks [%]	Reduction of reproduction compared to control [%]
Control	Control	4	-	397.2	-
32	19	6	2	355.6	10
50	29	6	2	384.6	3
100	59	2	-2	344.4	13
500	294	0	-4	446.4	-12
1000	587	8	4	358.8	10

[1] calculated with Abbott 1925

Reference test:

Treatment with the reference item ██████ (Phenmedipham EC 157 g/L) at concentrations of 50, 100, 200 and 400 mg test item/ kg dry soil resulted in significant effects on reproduction and the determined EC_{50} was 181.0 mg ██████/kg dry soil.

All validity criteria according to OECD 232 were fulfilled, since the mean adult mortality did not exceed 20%, the mean number of juveniles per vessel was ≥ 100 and the coefficient of variation of juveniles was less than 30%.

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III. CONCLUSION

In a 28 d laboratory test to determine the effects of MON 0139 on the collembolan, *Folsomia candida*, the 28-d EC_{50} was > 1000 µL MON 0139/ kg dry soil (>587 mg glyphosate acid equivalent/kg dry soil). The NOEC was 1000 µg/L test item/kg dry soil (587 mg glyphosate acid equivalent/kg dry soil), the highest tested concentrations, since MON 0139 had no negative effect on the test organisms.

Annex point	Author(s)	Year	Study title
IIA 8.9.2/07	[redacted]	2010	AMPA – Effects on the Reproduction of the Predatory Mite *Hypoaspis aculeifer* [redacted] Report No: 10 10 48 053 S Date: 2010-09-07 GLP: yes not published

Guideline: OECD 226 (2008)

Deviations: None

Dates of experimental work: 2010-05-28 to 2010-06-18

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Executive Summary

In the laboratory study the toxicity and reproductive inhibition of AMPA to *Hypoaspis aculeifer* was tested. Adult mites were exposed to 40, 80, 160, 240 and 320 mg test item/kg dry soil. In addition a blank control with deionised water and a toxic reference (Dimethoate EC 400) were tested.

40 mites (10/ test vessel) per test concentration and 80 mites per control (10/ test vessel) were put in a glass bottle on artificial soil with incorporated test item and adults and juveniles counted after 14 d. The test item AMPA caused no statistically significant mortality of adult *Hypoaspis aculeifer* at the end of the 14-day exposure period. Also no significant decrease in reproduction was observed. All validity criteria according to OECD 226 were fulfilled.

In conclusion, in a 14 d laboratory test to determine the effects of AMPA on the predatory mite, *Hypoaspis aculeifer*, the 14-d EC_{50} was > 320 mg test item/ kg dry soil. The NOEC was 320 mg test item/kg dry soil, the highest tested concentration, since AMPA had no negative effect on the test organisms.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: AMPA (Aminomethylphosphonic acid)
Description: White crystalline solid
Lot/Batch #: GLP-0908-19984-A

Purity:	98.7 %
2. Vehicle and/or positive control:	Reference item: Dimethoate EC 400 (414.8 g/L analysed)
3. Test organisms:	
Species:	*Hypoaspis aculeifer* (Canestrini)
Age:	Adult mites
Source:	[redacted] [redacted] Germany
Diet/Food:	*Tyrophagus putrescentiae* (Schrank) were fed every 2 days, before and during the test
4. Environmental conditions:	
Temperature:	19.7 – 21.8 °C
Composition of artificial soil	5% sphagnum peat 20% kaolin clay 0.3% calcium carbonate 74.7% quartz sand Deionised water
Soil water content:	Test start: 17.40 – 18.07% (47.81 – 49.64 % of WHC) Test end: 17.10 – 17.55% (46.98 – 48.22% of WHC)
pH	Test start: 5.8 – 6.1 Test end: 5.4 – 6.3
Photoperiod:	16 hours light / 8 hours darkness
Light intensity:	472 lux

B: STUDY DESIGN AND METHODS

1. Experimental treatments: AMPA was evaluated for mortality and reproductive reduction in a test with *Hypoaspis aculeifer* at five application rates, equivalent to 40, 80, 160, 240 and 320 mg test item/kg dry soil. In addition a blank control with deionised water and a toxic reference (Dimethoate EC 400) were conducted. Each test item concentration was tested with 40 mites (10/ test vessel), while the control group consisted of 8 replicates. For each test item concentration and for the control group 2 test vessels without mites were provided for measurement purposes. The mites were put in glass bottles with screw tops of 100 mL containing 20 g (dry weight) artificial soil with the requested test item concentrations and closed, but opened every second day for food supply and aeration. Two weeks after introducing the test organisms the parental and juvenile were counted.

2. Observations: Water content and pH were determined at test start and end. Adults and juvenile mites were counted at test end.

3. Statistical calculations: Fisher's Exact Binominal test with Bonferroni Correction for significance of parental mortality. Dunnett-t-test (α = 0.05) for significance of reproductive reduction. Statistical program: ToxRat Professional 2.10 (2009).

II. RESULTS AND DISCUSSION

A. FINDINGS

The EC_{50} value and the NOEC are given below based on nominal concentrations.

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Endpoints	AMPA [mg/kg dry soil]
NOEC	320
EC_{50} (14 d)	> 320

Reference test:

After treatment with the reference item Dimethoate EC 400 at concentrations of 4.1, 5.12, 6.40, 8.00 and 10.00 mg a.s./kg dry soil an EC_{50} (reproduction) of 6.6 mg test item/ kg dry soil was concluded.

B. OBSERVATIONS

The test item AMPA caused no statistically significant mortality (Fishers's Exact Binominal Test, $p > 0.05$) of the adult *Hypoaspis aculeifer* at the end of the 14-day exposure period. Also no significant decrease in reproduction was observed (Dunnett-t-test, $\alpha = 0.05$).

Table 8.9-7: Mortality and reproductive reduction of Hypoaspis aculeifer after application of AMPA in a 14 d laboratory study

Test rate [mg test item/kg dry soil]	Mortality of parental collembolans after 14days [%]	Corrected mortality [1] [%]	Mean number of juveniles after 14 days [%]	Reduction of reproduction compared to control [%]
Control	0.0	-	220.6	-
40	5.0	5.0	228.0	-3.3
80	2.5	2.5	236.3	-7.1
160	2.5	2.5	209.3	5.2
240	0.0	0.0	237.3	-7.5
320	2.5	2.5	227.5	-3.1

1 calculated with Abbott 1925

Reference test:

Treatment with the reference item Dimethoate EC 400 at a concentration of 4.1, 5.12, 6.40, 8.00 and 10.00 mg a.s./kg dry soil resulted in significant effects on reproduction.

All validity criteria according to OECD 226 were fulfilled, as adult mortality did not exceed 20%, the mean number of juveniles per replicate was > 50 at test end and the coefficient of variation of the number of juveniles per replicate was not higher than 30% at test end.

III. CONCLUSION

In a 14 d laboratory test to determine the effects of AMPA on the predatory mite, *Hypoaspis aculeifer*, the 14-d EC_{50} was> 320 mg test item/kg dry soil. The NOEC was 320 mg test item/kg dry soil, the highest tested concentration, since AMPA had no negative effect on the test organisms.

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Annex point	Author(s)	Year	Study title
IIA 8.9.2/08	[redacted]	2010	AMPA – Effects on the Reproduction of the collembolans *Folsomia candida* [redacted] Report No: 10 10 48 054 S Date: 2010-09-07 GLP: yes not published

Guideline: OECD 232 (2009)
ISO 11267 (1999)

Deviations: None

Dates of experimental work: 2010-06-01 to 2010-06-29

Executive Summary

In the laboratory study the toxicity and reproductive inhibition of AMPA to *Folsomia candida* was tested. Juvenile springtails, approximately 4 days old, were exposed to 30, 54, 97.2, 175 and 315 mg test item/kg dry soil. In addition a blank control with deionised water and a toxic reference (100% boric acid) were tested. 40 springtails (10/ test vessel) per test concentration and 80 springtails per control (10/ test vessel) were put in a glass container on artificial soil with incorporated test item and adults and juveniles counted after 28 d.

No statistically significant effects on parental mortality and number of offspring were observed. All validity criteria according to OECD 232 were fulfilled.

In conclusion, in a 28 d laboratory test to determine the effects of AMPA on the collembolan, *Folsomia candida*, the 28-d LC_{50} and EC_{50} were > 315 mg AMPA/kg dry soil. The NOEC was 315 mg test item/kg dry soil since AMPA had no negative effect on the test organisms.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: AMPA (Aminomethylphosphonic acid)
Description: White crystalline solid
Lot/Batch #: GLP-0908-19984-A
Purity: 98.7 %

2. Vehicle and/or positive control: Reference item: Boric acid (100%)

3. Test organisms:

Species: *Folsomia candida* (Willem)
Age: Juvenile springtails (9 – 12 d old)
Source: [redacted] Germany
Diet/Food: Approximately 2 mg granulated dry yeast at test start and after 14 days

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4. Environmental conditions:

Temperature:	20.4 – 22.0 °C
Composition of artificial soil	5% sphagnum peat 20% kaolin clay 0.3% calcium carbonate 74.6% quartz sand Deionised water
Soil water content:	Test start: 24.9 – 25.1% (57.8 – 58.2 % of WHC) Test end: 24.3 – 25.0% (56.4 – 58.0% of WHC)
Photoperiod:	16 hours light / 8 hours darkness
Light intensity:	750 lux

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: AMPA was evaluated for mortality and reproductive reduction in a test with *Folsomia candida* at five application rates, equivalent to 30, 54, 97.2, 175 and 315 mg test item/kg dry soil. In addition a blank control with deionised water and a toxic reference (100% boric acid) were conducted. Each test item concentration was tested with 40 springtails (10/ test vessel), while the control group consisted of 8 replicates. For each test item concentration and for the control group 2 test vessels without springtails were provided for measurement purposes. The springtails were put in a glass container (~ 150 mL) containing 30g (wet weight) artificial soil with the requested test item concentrations and covered with a glass lid for 28 d. Four weeks after introducing the test organisms the parental and juvenile were counted.

2. Observations: Water content and pH were determined at test start and end. Adults and juvenile springtails were counted at test end as well as physiological or pathological symptoms.

3. Statistical calculations: Fisher's Exact Binominal test with Bonferroni Correction for significance of parental mortality Dunnett-t-test ($\alpha = 0.05$) for significance of reproductive reduction Statistical program: ToxRat Professional 2.10 (1999).

II. RESULTS AND DISCUSSION

A. FINDINGS

The LC_{50} and EC_{50} values as well as the NOEC are given below based on nominal concentrations.

Endpoints	**AMPA [mg/kg dry soil]**
NOEC (mortality)	315
NOEC (reproduction)	315
LC_{50} (28 d)	> 315
EC_{50} (28 d)	> 315

Reference test:

After treatment with the reference item boric acid at concentrations of 44, 67, 97.2, 150 and 225 mg test item/ kg dry soil an EC_{50} of 108.6 mg test item/ kg dry soil and a NOEC for mortality and reproduction between 44 and 150 mg test item/kg dry soil were concluded. The LC_{50} could not be calculated but must be > 225 mg test item/ kg dry soil.

B. OBSERVATIONS

No statistically significant effects on parental mortality (Fisher's Exact Binominal Test, α = 0.05) or the number of offspring (Dunnett-t-test, α = 0.05) was found.

Table 8.9-8: Mortality and reproductive reduction of *Folsomia candida* after application of AMPA in a 28 d laboratory study

Test rate [mg test item/kg dry soil]	Mortality of parental collembolans after 4 weeks [%]	Corrected mortality [1] [%]	Mean number of juveniles after 4 weeks [%]	Reduction of reproduction compared to control [%]
Control	6.3	-	931	-
30	5.0	-1	925	1
54	7.5	1	934	0
97.2	2.5	-4	946	-2
175	7.5	1	973	-4
315	2.5	-4	939	-1

[1] calculated with Abbott 1925

Reference test:

Treatment with the reference item boric acid at concentrations of 44, 67, 97.2, 150 and 225 mg test item/ kg dry soil resulted in an EC_{50} of 108.6 mg test item/kg dry soil.

All validity criteria according to OECD 232 were fulfilled, since the mean adult mortality did not exceed 20%, the mean number of juveniles per vessel was ≥ 100 and the coefficient of variation of juveniles was less than 30%.

III. CONCLUSION

In a 28 d laboratory test to determine the effects of AMPA on the collembolan, *Folsomia candida*, the 28-d LC_{50} and EC_{50} were > 315 mg AMPA/ kg dry soil. The NOEC was 315 mg AMPA/kg dry soil, the highest tested concentrations, since AMPA had no negative effect on the test organisms.

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IIA 8.10 Effects on soil microbial activity

In the 2001 EU Evaluation of Glyphosate several laboratory soil micro-organism studies were reviewed. That evaluated the effect of glyphosate on microbial activities in soil. These tests were performed with SL formulations but one of these tests was performed with the active substance. It was concluded in the 2001 EU Evaluation of Glyphosate that when applying glyphosate containing products according to the mentioned amounts no negative effects on soil microbial activities are to be expected. Additionally published papers on glyphosate were cited that confirm that when applying glyphosate –based products according to the recommended pattern of use microflora will not be affected. A new study has been performed with the lead formulation, MON 52276, and the results for this study confirm no long-term adverse effects on soil microbial communities.

Only data from tests considered valid or studies considered in the previous evaluation are listed in the tables below. Further, only summaries of studies not considered in the 2001 EU Evaluation of Glyphosate are listed.

A summary of data reviewed for Annex I listing of glyphosate acid according to Directive 91/414/EEC is included in Table 8.10-1.

Table 8.10-1: Effects on soil micro-organisms

Compound	Study design	Endpoint	Reference/GLP	2001 EU evaluation monograph reference
Glyphosate acid	Nitrogen- and Carbon-mineralisation 28-day study	No significant effects (>25%) on nitrogen mineralisation by day 28 at 2.4 and 4.8 kg/ha, results for carbon mineralisation invalid	IIA 8.10.1/01 [redacted] D61.47/99 [redacted] 2000/yes	-
Glyphosate acid	Nitrogen- and Carbon-mineralisation 91/56-day study	No significant effects (>25%) on microbial respiration and nitrogen mineralisation at 2.16and 10.8 kg a.s./ha in loamy sand soil and loamy soil [2]		96-00006 [2]
MON 52276	Nitrogen- and Carbon-mineralisation 28-day study	No significant effects (>25%) on dehydrogenase activity and nitrogen mineralisation by day 28 at 18.8 and 94 mg MON 57726/kg dry soil, (12 and 60 L MON52276/ha) in loamy sand	IIIA 10.7.1/01 [redacted]-5259 [redacted] 2011/yes	-
AMPA	Nitrogen- and Carbon-mineralisation 28/56-day study	No significant effects (>25%) on carbon transformation and nitrogen mineralisation by day 28 at concentrations of up to 160 mg/kg dry soil	IIA 8.10.1/02 10 10 48 010 C/N [redacted] 2010/yes	-

[1] Study was performed with unspecified formulation containing 360 g glyphosate/L.
[2] As a general indication, extremely variable control values suggest an invalid test design

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IIA 8.10.1 Nitrogen transformation

Annex point	Author(s)	Year	Study title
IIA 8.10.1/01	[redacted].	2000	Side-Effects of Glifosate Técnico Nufarm on Soil Microflora Carbon and Nitrogen Cycles [redacted] Report No: [redacted]-D61.47/99 Report No: [redacted]-D1. 113/99 Date: 2000-01-05 GLP: yes not published

Guideline: Instituto Brasileiro do Meio ambiente e dos Recursos naturais Renovaveis Ibama, portaria Normativa no 84 of October, 15 1996

Deviations from OECD 216 and 217: For soil nitrogen cycle (OECD 216) test the variation between replicate control samples was more than ± 15%.

Dates of experimental work: 1999-10-10 to 1999-11-12

Executive summary

The effects of glyphosate acid on soil carbon cycle and nitrogen cycle were investigated in two soil types, a “Typic Hapludox” and a “Rhodic Hapludox” in laboratory conditions. The test substance was applied at two concentration rates of 2.4 and 4.8 kg a.s./ha in three replicates. In addition, negative controls (without test item) with or without organic matter amendment were tested. 150 g soil samples were amended with organic matter at a rate of 0.5% dry soil equivalent for all treatments, except for control without organic matter amendment. Soils were incubated at a temperature range of 19 to 22°C in dark in covered glass flasks. Soil samples were removed from the jars 0, 14 and 28 days after treatment and analysed for soil dry mass, pH, nitrite, nitrate, nitrogen, ammonical and short term respiration.

The results showed no adverse effects of glyphosate acid on soil carbon cycle for both concentrations tested, 28 days after application. In addition, all validity criteria according to OECD 217 were fulfilled for carbon cycle study. For soil nitrogen cycle test however, the validity criteria according to OECD 216 were not fulfilled, as the variation between replicate control samples were more than ± 15%.Therefore, no consistent conclusions could be drawn from the study.

The test item glyphosate acid caused no adverse effects on soil carbon cycle a test concentration of 2.4 and 4.8 kg a.s/ha, 28 days after treatment. The nitrogen cycle test is considered not valid according to OECD guideline 216.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: White powder
Lot/Batch #: 037-919-113
Purity: 95% a.s (nominal), 95.49% a.s. (measured)

2. Vehicle and/or positive control: None

3. Test system:

Soil	LE (Typic Hapludox) and LR (Rhodic Hapludox)
Source:	Not stated
Water content of soil:	Not stated
Water holding capacity	Not stated
pH:	5.5(LR), 7.0(LE)
Total Org. C:	Not stated
Clay (< 0.002 mm):	39 %(LR), 24%(LE)
Silt (0.002 mm - 0.063 mm):	10% (LR), 9%(LE)
Sand (0.063 – 2.00 mm):	51% (LR), 67%(LE)

4. Environmental conditions:

Temperature:	19 - 22°C
pH:	5.53 – 6.27 (LR), 6.34 – 6.84 (LE)
Water content:	40- 60% of WHC
Photoperiod:	24 hour dark

B. STUDY DESIGN AND METHODS

1. Experimental treatments: The test substance was applied at two concentration rates of 2.4 and 4.8 kg a.s./ha using three replicates per concentration. In addition, negative controls (without test item) with or without organic matter amendment were tested. 150 g soil samples were amended with organic matter at a rate of 0.5% dry soil equivalent for all treatments, except for control without organic matter amendment. Soils were incubated at a temperature range of 19 to 22°C in dark in covered glass flasks. Soil samples were removed from the jars, 0, 14 and 28 days after treatment and analysed for soil dry mass, pH, nitrite, nitrate, nitrogen ammonical and short term respiration test.

2. Observations:

Nitrogen cycle: For the preparation of soil extract for ammonium-N analysis, 10 g of soil was placed in 250 mL wide-mouth bottle, to which 100 mL of 2M KCl was added. 1 mL of the filtered aliquot containing between 0.5 and 12 µg of NH_4+-N was placed into 25 mL volumetric flasks. 1 mL EDTA, 2 mL phenol nitroprussid and 4 mL hypochlorite buffer were successively added. The concentration of NH_4+-N was thereafter determined using a photometric method at 636 nm. For nitrate-N and nitrite -N analysis, 10 g of soil was placed in a 500 mL Erlenmeyer flask, then 0.5 g of $CaSO_4$ and 250 mL distilled water were added. For the analysis of nitrate-N, an aliquot of 25 mL of the extract was pipetted into 10 mL round bottom flask and 0.05g of $CaCO_3$ was added. Subsequently, 2 ml of phenoldisulfonic acid (25 g phenol in 150 mL of concentrated NH_4OH) was added. After 10 min, 20 ml of distilled water was added. The nitrate-N concentration was determined using a Hach Model DR 2010 absorbance spectrophotometer at 410 nm. For the analysis of nitrite-N, an aliquot of 25 mL of the extract was pipetted into a 25 mL cell. The visual absorbance of each sample was determined at 507 nm using a Hach Model DR 2010 absorbance spectrophotometer.

Carbon cycle: 2 g of soil samples were placed in 50 mL Erlenmeyer flasks, adding 0.5 mL of 2 µmol/mL of glucose- ^{14}C. In order to absorb CO_2 evolved from glucose degradation by soil microorganisms, a small glass flask (1 mL) was hung from the rubber cap, containing 0.2 mL of NaOH. After one hour of incubation in dark conditions, the glucose degradation was then stopped. The NaOH and filter paper strips were transferred into scintillation vials. The radioactivity was assessed in a Liquid Scintillation Analyser Packard model Tri-carb 1900, during 5 min/sample.

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3. Statistical calculations: Results were evaluated using Duncan's Multiple range Test at $\alpha = 0.01$.

II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

No adverse effects of glyphosate acid on soil carbon cycle were observed for both concentrations 28 days after application. In addition, all validity criteria according to OECD 217 were fulfilled. For soil nitrogen cycle test validity criteria according to OECD 216 were not fulfilled, as the variation between replicate control samples was more than ± 15%.

Table 8.10.1-1: Effects of glyphosate acid on soil nitrogen cycle

		Glyphosate acid [kg a.s./ha]				
		Control	**2.4**		**4.8**	
		[mg N/kg dry soil]	**[mg N/kg dry soil]**	**Dev. [a]**	**[mg N/kg dry soil]**	**Dev. [a]**
Soil: LR (Rhodic Hapludox)						
Day 0	Ammonium	22.66	21.61	-4.6	24.31	+7.3
	Nitrite	0.30	0.29	-3.3	0.40	+33.3*
	Nitrate	22.51	22.54	+0.1	23.11	+2.7
Day 14	Ammonium	27.34	34.92	+27.7*	37.50	+37.2*
	Nitrite	0.29	0.21	-27.6*	0.23	-20.7
	Nitrate	30.02	36.47	+21.5*	44.10	+46.9*
Day 28	Ammonium	13.13	11.32	-13.8	9.38	-28.6*
	Nitrite	0.26	0.24	-7.7	0.24	-7.7
	Nitrate	18.39	24.16	+31.4*	34.61	+88.2*
Soil: LE (Typic Hapludox)						
Day 0	Ammonium	30.01	27.87	-7.1	34.72	+15.7
	Nitrite	0.32	0.27	-15.6	0.27	-15.6
	Nitrate	22.58	22.74	+0.7	23.34	+3.4
Day 14	Ammonium	26.19	22.60	-13.7	24.50	-6.5
	Nitrite	0.26	0.29	+11.5	0.27	+3.8
	Nitrate	21.78	39.26	+80.3*	41.01	+88.3*
Day 28	Ammonium	16.82	18.71	+11.2	18.72	+11.3
	Nitrite	0.40	0.24	-40.0*	0.26	-35.0*
	Nitrate	18.39	31.67	+72.2*	25.77	+40.1*

[a] - = Deviation from control
* = Significant deviation from control according to OECD Guideline 216 and 217
- = inhibition, + = stimulation

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Table 8.10.1-2: Effects of glyphosate acid on soil carbon cycle

	Glyphosate acid [kg a.s./ha]				
	Control	2.4		4.8	
	Soil respiration [b]	Soil respiration [b]	Dev. [a]	Soil respiration [b]	Dev. [a]
Soil: LR (Rhodic Hapludox)					
Day 0	9.00	8.33	-7.4	9.06	+0.7
Day 14	16.06	16.19	+0.8	16.76	+4.4
Day 28	15.13	14.63	-3.3	16.53	+9.3
Soil: LE (Typic Hapludox)					
Day 0	12.80	13.00	+1.6	11.56	-9.7
Day 14	16.69	20.16	+20.8	17.56	+5.2
Day 28	16.43	18.06	+9.9	17.26	+5.1

[a] - = Deviation from the control based on NO_3-nitrogen production
[b] = Activity of soil microorganism in mmoles metabolized glucose/g soil/h
- = inhibition, + = stimulation

III. CONCLUSION

The test item glyphosate acid caused no adverse effects on soil carbon cycle a test concentration of 2.4 and 4.8 kg a.s/ha, 28 days after treatment. The nitrogen cycle test is considered not valid according to OECD guideline 216.

Annex point	Author(s)	Year	Study title
IIA 8.10.1/02	████	2010	AMPA - Effects on the Activity of Soil Microflora (Nitrogen and Carbon Transformation Tests) ████ ████ Report No: 10 10 48 010 C/N Date: 2010-10-07 GLP: yes not published

Guideline: OECD 216 (2000)
OECD 217 (2000)

Deviations to OECD 216 and 217: None

Dates of experimental work: 2010-05-20 to 2010-07-15

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Executive summary

The effects of AMPA on soil nitrogen transformation and soil carbon transformation were investigated in a loamy sand soil. The test substance was applied at concentration rates of 40, 80, 160, 320 and 640 mg AMPA/kg dry soil using three replicates per treatment. In addition, a negative control (non-treated soil) was tested.

The results showed no adverse effects of the test item 28 days after application on nitrogen and carbon transformation in soil up to and including a test concentration of 160 mg AMPA/kg dry soil. Due to measured deviations of > 25 % observed in the treatment groups treated with 320 and 640 mg AMPA/kg dry soil, 28 days after application, the test was prolonged to 56 days for both treatment levels. After a test

prolongation, the measured variations of nitrogen and carbon transformations of > 25% could be observed till the end of the study (56 days). All validity criteria according to OECD 216 and 217 were fulfilled.

The test item AMPA caused no adverse effects on soil nitrogen transformation and on soil carbon transformation up to and including a test concentration of 160 mg AMPA/kg dry soil at the end of 28-days incubation period.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	AMPA (Aminomethylphosphonic acid)
Description:	White crystalline solid
Lot/Batch #:	GLP-0908-19984-A
Purity:	98.7%
2. Vehicle and/or positive control:	Reference item: Dinoterb
3. Test system:	
Soil	Loamy sand soil "Wassergut Canitz" (agricultural soil)
Source:	Field [redacted] Germany.
Water content of soil:	11.30% (g water/100 g dry soil)
Water holding capacity	36.56% (g water/100 g dry soil)
pH:	6.3
Total Org. C:	1.43%
Clay (< 0.002 mm):	9.1 %
Silt (0.063 mm > 0.002 mm):	38.2%
Sand (≥ 0.063 – 2.00 mm):	52.7%
4. Environmental conditions:	
Temperature:	19.7 – 21.8°C
pH:	5.9 – 6.3
Water content:	41.46 – 44.71% of WHC (nitrogen transformation test) 41.84 – 45.09% of WHC (carbon transformation test)
Photoperiod:	24 hours darkness

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B. STUDY DESIGN AND METHODS

1. Experimental treatments:

AMPA was applied at concentration rates encompassing 40, 80, 160, 320 and 640 mg test item/kg dry soil. In addition, a negative control (non-treated soil) was tested. Three replicate soil samples were prepared for each treatment rate and the control for the carbon transformation and nitrogen transformation tests.

Soil carbon transformation: For each replicate a sub-sample of 1000 g dry soil was mixed with deionised water. Water was added to the soil to achieve a water content of approximately 45% WHC. Water content

was adjusted weekly to the required range of 40-50% of WHC. The prepared soil was transferred to steel test vessels (4 L) under 19.7 – 21.8°C in a climatic room.

Soil nitrogen transformation: Sub-samples of 200 g dry soil were weighed into each test vessel (500 mL wide mouth glass flask). Lucerne meal (5 g/kg dry soil) was then added to provide 1.0 g Lucerne meal per 200 g dry soil. One additional soil sample (without Lucerne meal) was used for determination of initial NH_4-N- and NO_3-N-content. The initial NH_4-N and NO_3-N content was 0.01 mg and 1.48 mg/100 g dry soil, respectively. Incubation of the prepared soil was carried out in wide-mouth glass flasks (500 mL) under 19.7 – 21.8°C in a climatic room.

2. Observations:

Soil carbon transformation: Carbon transformation was determined for a measurement period of 12 hours on sampling days 0 (3 hours after application), 7, 14, 28, 42 and 56 days after application On each sampling occasion, 100 g samples of soil (dry soil) were taken, mixed with glucose by means of a hand-stirrer and placed into glass reaction flasks (500 mL). Then, glass vessels containing 18 mL of 1 M NaOH solution were placed in the reaction flasks and connected with a respirometer ( ). Cumulative oxygen production (corresponding to the O_2 consumption by micro-organisms) was determined over a 12-hour measurement period.

Soil nitrogen transformation: Soil samples (10 g dry soil per replicate) were sampled at intervals of 3 hours, 7, 14, 28, 42 and 56 days after application and NH_4-N, NO_3-N and NO_2-N contents were determined. Soil was extracted by adding 50 mL 1 M KCl solution to the equivalent of 10 g dry soil. Quantitative determination of mineralized nitrogen was performed using an Autoanalyzer II.

3. Statistical calculations: Two-sided Students t-test for homogenous variances at $\alpha = 0.05$. For carbon transformation, a two-sided Welch t-test for homogenous variance was additionally performed.

II. RESULTS AND DISCUSSION

A. FINDINGS AND OBSERVATIONS

28 days after application, no adverse effects on nitrogen and carbon transformation were observed up to and including a test concentration of 160 mg AMPA/kg dry soil. After the prolongation of the test to 56 days for the test concentrations 320 and 640 mg AMPA/kg dry soil, the measured variations of nitrogen and carbon transformations of > 25% could be observed till the end of the study (56 days). This can be most likely attributed to the high phosphorus/nutrient content in AMPA.

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Table 8.10.1-3: Effects of AMPA on soil nitrogen transformation

	AMPA [mg test item/kg dry soil]										
	Control	40		80		160		320		640	
	NO_3-N	NO_3-N	Dev. [a]	NO_3-N	Dev. [a]	NO_3-N	Dev. [a]	NO_3-N	Dev. [a]	NO_3-N	Dev. [a]
Day 0	15.7	15.5	-1.1	15.7	2	15.4	-1.9	14.9*	-4.9	14.6*	-6.6
Day 7	23.1	23.6	2.5	27.3*	18.5	25.8	+11.7	30.5*	+32.2	33.5*	+45.2
Day 14	32.2	34.6	7.5	37.4*	16.3	35.1*	+9.2	42.9*	+33.3	43.9*	+36.5
Day 28	42.2	46.8*	10.7	47.7*	13	51.0*	+20.8	57.4*	+35.8	65.0*	+53.8
Day 42	55.4	-	-	-	-	-	-	72.1*	+30.2	78.1*	+41.1
Day 56	61.9	-	-	-	-	-	-	78.4*	+26.7	88.6*	+43.1

[a] - = Deviation from the control based on NO_3-nitrogen production
* = Significantly different from control (two-sided Student- t test for homogenous variances at $\alpha = 0.05$)
- = inhibition, + = stimulation

Table 8.10.1-4: Effects of AMPA on soil carbon transformation

	AMPA [mg test item/kg dry soil]										
	Control	40		80		160		320		640	
	O_2[a]	O_2[a]	Dev. [b]	O_2[a]	Dev. [b]	O_2[a]	Dev. [b]	O_2[a]	Dev. [b]	O_2[a]	Dev. [b]
Day 0	12.0	11.9	-0.8	11.4*	-5.3	11.1*	-8.0	10.8*	-10.4	10.1*	-16.2
Day 7	11.9	11.0*	-7.1	10.3*	-13.2	9.9*	-16.9	9.5*	-20.2	8.3*	-29.7
Day 14	11.7	10.9*	-7.0	10.6*	-9.2	9.9*	-15.4	9.1*	-22.6	8.0*	-31.3
Day 28	10.9	10.0*	-7.9	9.5*	-12.9	8.9*	-18.5	8.1*	-25.7	7.0*	-35.3
Day 42	10.7	-	-	-	-	-	-	7.9*	-26.6	6.8*	-37.0
Day 56	10.1	-	-	-	-	-	-	7.4*	-26.1	6.2*	-38.8

[a] - = Oxygen consumption
[b] - = Deviation from the control based on NO_3-nitrogen production
* = Significantly different from control (two-sided Student- t test or two-sided Welch-t-test, respectively for homogenous variances at $\alpha = 0.05$)
- = inhibition, + = stimulation

In a different test, 28 days after application the toxic standard dinoterb caused effects of +37.6%, +51.4% and +27.1% on nitrogen transformation and -30.5%, -34.5% and -28.8% on carbon transformation at concentrations of 6.80, 16.0 and 27.0 mg dinoterb/kg dry respectively, and thus demonstrates the sensitivity of the test system.

All validity criteria according to OECD 216 and 217 were fulfilled, as the variation between replicate control samples was less than ± 15%.

III. CONCLUSION

The test item AMPA caused no adverse effects on soil nitrogen transformation and on soil carbon transformation (< 25% deviation from control) up and including a test concentration of 160 mg AMPA/kg dry soil at the end of 28-day incubation period.

IIA 8.10.2 Carbon mineralization

Nitrogen transformation and carbon mineralization tests were conducted in the same studies. The study endpoints are summarised in Point IIA.8.11 in this document.

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IIA 8.10.3 Rates of recovery following treatment

As glyphosate does not pose an unacceptable risk to soil microflora, further testing is not required.

IIA 8.11 Effects on marine and estuarine organisms

This is not an EU data requirement.

However, several studies conducted with *Skeletonema costatum*, a marine plankton diatom are available and listed under Section IIA 8.4.

IIA 8.11.1 Marine or estuarine organisms acute toxicity LC_{50}/EC_{50}

No EC data requirement.

IIA 8.11.2 Marine/estuarine fish – salinity challenge

No EC data requirement.

IIA 8.12 Effects on terrestrial vascular plants

At the time of the previous Annex I inclusion, no risk assessment for terrestrial vascular plants was required. Therefore, no studies are presented within the scope of Commission Document 6511/VI/99-final dated 21 January 2002. However, the vegetative vigour study by [redacted] (1994) was reviewed and the Tier 2 summary has been included for completeness. Additionally, a Tier 2 summary for a vegetative vigour study with MON 52276 has been included as confirmatory data. The studies presented for the non-target plant assessment meet current OECD 227 guideline requirements.

However, two studies on seed germination and seedling emergence and one study on vegetative vigour have been evaluated within the scope of the 2001 EU-Evaluation of Glyphosate. In a non-GLP study, [redacted] (1987, ARW 97-00101) exposed seven dicotyledonous and four monocotyledonous weed species to 5.7 and 11.2 kg a.s./ha and observed no effects on any species except on *Ipomoea sp.* (morning glory) and *Polygonum pensylvanicum* (Pennsylvania smartweed), two species not occurring in Europe. [redacted] exposed 10 different crops to glyphosate at application rates at up to 5.1 kg a.s./ha (see Table 8.12-1). [redacted] 1989 (cited in Mensink & Janssen, ARW 97-00100, Glyphosate, Environmental Health Criteria monograph No. 159, Geneva: World Health Organization, 1994) exposed native British plant species to spray drift at several distances downwind (2.5 and 3.5 m/s) from a zone sprayed with 0.5 and 2.2 kg a.s./ha. Damage occurred at a distance of 2-6 m from the sprayer, but most of the damaged plants recovered. Most sensitive species were *Digitalis purpurea*, *Centaurea nigra*, *Prunella vulgaris* and *Lychnis flos-cuculi*. The authors concluded that, when spraying with ground sprayers, buffer zones around nature reserves should be 5-10 m.

A summary of the endpoints of the most sensitive species is presented in Table 8.12-1 whilst full details of these studies are provided below.

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Table 8.12-1: Toxicity of glyphosate and formulation MON 52276 to non-target plants

Test substance Test type	Most sensitive species	Lowest ER_{50}	Reference/GLP
Glyphosate acid 21 d vegetative vigour	*Helianthus annuus* (sunflower)	ER_{50} (dry weight) = 295.9 g a.s./ha	IIA 8.12/01 236 GLY , 1994/yes
Glyphosate acid 21 d vegetative vigour	*Solanum lycopersicum* (tomato)	ER_{50} (dry weight) = 145.7 g a.s./ha	IIA 8.12/02 -13320 1994/yes Monograph reference 97-00102
Glyphosate acid (formulated product, WP) 28 d vegetative vigour	Oilseed rape (*Brassica napus*)	ER_{50} (visual damage) = 140 g a.s./ha	IIA 8.12/03 2009B 1996/yes
MON 52276 22 d vegetative vigour	Garden cress (*Lepidum sativum*)	ER_{50} (fresh weight) = 252 g a.s/ha	IIA 8.12/05 .104 2005
Glyphosate acid (formulated product, WP) 28 d Seedling emergence	Purple nutsedge (*Cyperus rotundus*) Oat (*Avena sativa*) Winter wheat (*Triticum aestivum*) Maize (*Zea mays*) Onion (*Allium cepa*) Sugar beet (*Beta vulgaris*) Lettuce (*Lactuca sativa*) Oilseed rape (*Brassica napus*) Cucumber (*Cucumis sativa*) Soybean (*Glycine max*) Okra (*Abelmoschus esculentus*) Rhubarb (*Rheum rhoponticum*)	ER_{50} (seedling emergence, seedling dry weight) > 4.48 kg a.s/ha	IIA 8.12/04 2008B 1996/yes

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Annex point	Author(s)	Year	Study title
IIA 8.12/01		1994	LX1146-02 (Glyphosate Technical) Tier II Non-Target hazard Evaluation – Terrestrial Vegetative Vigor Report No: 236 GLY Date: 1994-07-22 GLP: yes Not published

Guideline: EPA Guidelines, Subdivision J, Series 123-1 (b)

Deviations: None

Dates of experimental work: 1993-05-24 to 1993-06-25 (first test)
1993-09-29 to 1993-10-22 (test continuation)

Executive Summary

A vegetative vigour study was conducted exposing six dicotyledonous (carrot, cucumber, radish, soybean, sunflower, tomato) and four monocotyledonous (field corn, oat, onion, wheat) plant species to five nominal test concentrations of glyphosate, encompassing 0.0056, 0.0112, 0.0235, 0.0471 and 0.0930 kg glyphosate acid/ha in four replicates per treatment. In addition, a negative control group treated with deionized water was tested. The application was performed using a single nozzle hand-held, CO_2 pressurized sprayer. Because of poor rate response in most crops, five additional treatment rates were included, encompassing 0.0930, 0.1861, 0.3721, 0.5582 and 0.7442 kg glyphosate acid/ha.

Seedling number and plant height were recorded 7 days before treatment, on the day of treatment, 14 days after treatment (DAT) and 21 DAT. For the dry weight measurements, plants within a treated replicate were harvested 21 DAT and dried for a minimum of 24 h at approximately 100°C. Plant survival observations were recorded 7 DAT (6 DAT for the continuation test), 14 DAT (13 DAT for the continuation test) and 21 DAT. Phytotoxicity was evaluated 7, 14 and 21 DAT for initial test and 6, 13 and 21 DAT for the continuation test.

Plant height, plant dry weight and survival were significantly affected by glyphosate treatments in all species tested. Among monocotyledonous species, oat was most tolerant to glyphosate, while all other species exhibited approximately the same level of sensitivity to glyphosate. Among dicotyledonous species, sunflower and radish were most sensitive for glyphosate, whilst tomato, carrot and soybean showed a moderate sensitivity to glyphosate. Cucumber was the most tolerant species to glyphosate. For phytotoxicity, monocots and dicots were also affected by glyphosate treatments. The validity criteria according to the OECD 227 were fulfilled, except the fact that no data on seedling emergence in control group were reported.

The lowest (worst case) 21 day ER_{50} values of glyphosate were observed for sunflower plants and were calculated to be 0.3508, 0.2959 and 0.2993 kg glyphosate acid/ha for survival, dry weight and plant height, respectively. The lowest 21-day NOER value was observed for plant height and visual phytotoxicity and was determined to be 0.0930 kg glyphosate acid/ha.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Description: Solid, white

Lot/Batch #: 206-JAK-119-1

Purity: 98.5% (technical)

2. Vehicle and/or positive control: None

3. Test organism:

Species: 6 Dicotyledons: (carrot[1], cucumber[1], radish[1], soybean[3], sunflower[2], tomato[1])
4 Monocotyledons: (field corn[2], oat [2], onion[1], wheat[2])

Source: 1. .
2.
3.

4. Environmental conditions:

Temperature: Approx. 12.2°C – 37.8°C

Relative humidity: 70% - 94%

Photoperiod: 10 h light / 14 h dark , approx. 5443– 42531 Lux

Soil pH: 5.5 - 5.6

Soil organic matter content: 0.94 - 1.5%

B. STUDY DESIGN AND METHODS

1. Experimental treatments: Prior to treatment, seedlings were grown in plastic pots (approx.15 cm round) containing approximately 1 kg of pasteurised sandy soil. Small seeds (carrot, onion, radish and tomato) were planted at a depth of 0.5 to 1 cm and large seeds (field corn, wheat, oat, cucumber, sunflower and soybean) were planted at a depth of 1 to 1.5 cm. Soybean seeds were inoculated with commercial *Rhizobium japonicum*. Four replicate pots for each treatment were prepared for each species tested. At least 7 days prior to application, seedlings were grown to 1-3 true leaves and then thinned to five plants per replicate and their height recorded. The plants were treated with 5 nominal concentrations (adjusted to test item purity), encompassing 0.0056, 0.0112, 0.0235, 0.0471 and 0.0930 kg glyphosate acid/ha. In addition, one negative control group (treated with deionized water) was tested. Application was performed using a single nozzle hand-held CO_2 pressurized sprayer, starting with the water control. Plants were not watered during the first 24-hour period to avoid wetting the plants foliage and dislodging spray residue. Because of poor rate response in most crops, a test continuation was initiated at five additional concentration rates, encompassing 0.0930, 0.1861, 0.3721, 0.5582 and 0.7442 kg glyphosate acid/ha.

2. Observations: Seedling survival and plant height were recorded 7 days before treatment (DBT), on the day of treatment, 14 days after treatment (DAT) and 21 DAT. For dry weight measurements, plants were harvested 21 DAT and dried for a minimum of 24 h at approximately 100°C. Plant survival observations were recorded 7 DAT (6 DAT for the continuation test), 14 DAT (13 DAT for the continuation test) and 21 DAT. Phytotoxicity was evaluated 7, 14 and 21 DAT for initial test and 6, 13 and 21 DAT for the continuation test.

3. Statistical calculations: Data were analysed using two-way ANOVA and an LSD test was performed as post-hoc. The actual ER_x values were estimated by regression analysis using Lotus 1,2,3 Software.

II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

Plant height, dry weight and survival: Height, dry weight and survival of plants were significant affected by glyphosate treatments in all species tested. Among the monocotyledonous species, oat was most

tolerant to glyphosate while all other species exhibited approximately the same level of sensitivity to glyphosate. Among the dicotyledonous species, sunflower and radish were the most sensitive species, whilst tomato, carrot and soybean exhibited moderate sensitivity to glyphosate. Cucumber was the most tolerant species to glyphosate. For phytotoxicity, onion was the most tolerant monocot while other monocots tested showed approximately the same level of sensitivity to glyphosate.

Visual phytotoxicity: Visual phytotoxicity was generally expressed within 13 days after the treatment and did not substantially increase by 21 days. Onion exhibited tip burn (necrosis at the leaf tip and margins) at 0.7442 kg a.s./ha but no visual phytotoxicity at any of the lower rates. Oat exhibited visual phytotoxicity at a rate of 0.3721 kg a.s./ha, whereas wheat and field corn showed signs of visual phytotoxicity at rates as lower as 0.1861 kg a.s./ha. Glyphosate caused multiple shoots to develop at the soil line; higher application rates caused necrosis at the leaf tips. Despite the levels of visual injury observed on field corn, wheat and oat for all concentration tested, the plant height and dry weight were not significantly affected by glyphosate treatments.

For dicots, visual phytotoxicity occurred within 13 DAT and did no increase significantly by 21 days. When comparing the 21-day data, carrot was the most tolerant dicot with a NOER of 0.3721 kg glyphosate acid/ha and exhibited no phytotoxicity at rates below 0.5582 kg a.s./ha. The only injury observed from the glyphosate was slight chlorosis and stunting for carrot. With the exception of soybean (NOER = 0.1861 kg glyphosate acid/ha),the NOER for dicots was 0.0930 kg glyphosate acid/ha. For radish and sunflower, mortality was observed at the two highest rates tested and significant treatment effects were also noted in plant height and dry weight.

The resulting ER_{50} and NOER values for glyphosate acid are presented in the table below.

Crop	Endpoint [kg glyphosate acid/ha]					
	Survival		Dry weight		Plant height	
	ER_{50}	NOER	ER_{50}	NOER	ER_{50}	NOER
Onion	> 0.7442	0.7442	n.d.	0.0930	> 0.7442	0.0930
Field corn	> 0.7442	0.7442	0.6400	0.0930	> 0.7442	0.0930
Oat	> 0.7442	0.7442	>0.7442	0.7442	>0.7442	0.7442
Wheat	> 0.7442	0.7442	0.6478	0.0930	>0.7442	0.0930
Soybean	> 0.7442	0.7442	0.6758	0.1861	0.6590	0.1861
Radish	0.2488	0.0930	0.2623	0.0930	0.6904	0.0930
Cucumber	> 0.7442	0.7442	> 0.7442	0.7442	> 0.7442	0.1861
Sunflower	0.3508	0.1861	0.2959	0.0930	0.2993	0.0930
Tomato	> 0.7442	0.7442	0.5335	0.1861	> 0.7442	0.0930
Carrot	> 0.7442	0.7442	0.6512	0.3721	> 0.7442	0.1861

n.d. = not determined

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Table 8.12-2: Effects of glyphosate acid on height, dry weight and survival of non-target plants (test continuation, all species)

Crop	Glyphosate [kg glyphosate acid /ha]				
	0.0930	**0.1861**	**0.3721**	**0.5582**	**0.7442**
Mean plant height (cm)[% deviation from control]					
Onion	-20.34	-20.67	-13.03	-10.67	-32.53
Field corn	2.50*	-15.48	-15.94	-28.17	-44.76
Oat	6.50	13.93	9.68	1.72	0.27
Wheat	-4.77*	-22.43	-22.98	-23.77	-37.89
Soybean	5.41	-5.41*	-35.33	-48.36	-49.72
Radish	-14.64*	-33.67	-23.16	-100.00	-100.00
Cucumber	5.66	-7.03*	-27.96	-28.53	-32.86
Sunflower	25.92*	-47.28	-62.93	-100.00	-100.00
Tomato	-1.49*	-17.54	-28.73	-30.60	-43.28
Carrot	0.48	-12.28*	-22.66	-35.34	-40.62
Mean plant dry weight (mg/plant)[% deviation from control]					
Onion	-39.06	-50.00	-12.50	3.13	-34.38
Field corn	-5.83	-24.27	-33.01	-45.63	-53.88
Oat	5.77	-9.62	-13.46	-20.19	-11.06*
Wheat	-18.33*	-34.58	-50.00	-45.28	-45.14
Soybean	-8.90	-10.99*	-33.51	-46.86	-49.21
Radish	-29.07	-54.46	-57.36	-100.00	-100.00
Cucumber	12.60	13.39	11.81	20.73	10.43*
Sunflower	0.00*	-50.22	-57.24	-100.00	-100.00
Tomato	-18.10	-11.21*	-44.83	-55.17	-62.93
Carrot	13.04	33.70	30.43*	46.74	50.72
Mean plant survival 21 DAT[% deviation from control]					
Onion	-5.00	0.00	0.00	-5.00	-5.00
Field corn	0.00	0.00	0.00	0.00	0.00
Oat	0.00	0.00	0.00	-5.00	-5.00
Wheat	0.00	-5.00	0.00	-15.00	-20.00
Soybean	0.00	0.00	0.00	-5.00	0.00
Radish	0.00*	-40.00	-80.00	-100.00	-100.00
Cucumber	0.00	0.00	-10.00*	-40.00	-20.00
Sunflower	0.00	-25.00*	-55.00	-100.00	-100.00
Tomato	0.00	0.00	0.00	0.00	0.00
Carrot	5.26	0.00	5.26	-5.26	-5.26

* = significantly different when compared to the control ($\alpha = 0.05$)

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Table 8.12-3: Effects of glyphosate acid on plant height and dry weight and survival (initial test, onion and radish)

Crop	Glyphosate acid [kg/ha]				
	0.0056	**0.0112**	**0.0235**	**0.0471**	**0.0930**
Mean plant height [cm]					
Onion	-2.68	-10.92	-15.52	-11.30	-20.31
Mean plant dry weight [mg/plant]					
Onion	-19.23	-26.92	-19.23	-13.46	-28.85
Radish	-33.33	-20.99	-23.46	33.33	-4.94

The validity criteria according to the OECD 227 were fulfilled, except the fact that no data on seedling emergence in control group were reported.

III. CONCLUSION

The lowest (worst case) 21 day ER_{50} values of glyphosate were determined for sunflower and were calculated to be 0.3508, 0.2959 and 0.2993 kg glyphosate acid/ha for survival, dry weight and plant height, respectively. The lowest 21-day NOER value was observed for plant height and visual phytotoxicity and determined to be 0.0930 kg glyphosate acid/ha.

Annex point	Author(s)	Year	Study title
IIA 8.12/02	███	1994	Tier 2 Vegetative Vigor Nontarget Phytotoxicity Study Using Glyphosate ███ Report No: ███-13320 Date: 1994-01-14 GLP: yes Not published

Guideline: OECD Guideline 227 (2006)
EPA Guidelines, Subdivision J, Series 123-1 (b)

Deviations TO OECD 227: None

Dates of experimental work: 1993-06-16 to 1993-09-03

Executive Summary

A Tier 2 vegetative vigour study using glyphosate acid and a non-ionic surfactant was conducted on 6 dicotyledonous and 4 monocotyledonous species: soybean, lettuce, radish, tomato, cucumber, cabbage, oat, ryegrass, corn and onion. The test material was applied to plants at the 1 to 3 leaf stage at rates ranging from 0.078 to 5.04 kg a.s./ha. Radish and tomato were also tested at 5 additional rates ranging from 0.005 to 0.078 kg a.s./ha. Radish and tomato were also tested at 5 additional rates ranging from 0.005 to 0.078 kg a.s./ha. Phytotoxicity observations were recorded at 7, 14 and 21 days after treatment. Dry weight and plant height were determined 21 days after treatment. The most sensitive species tested were tomato and radish. Based on ER_{50} data, the most sensitive parameter among those analysed was dry weight, with ER_{50} values of 0.146 kg a.s./ha for tomato and 0.246 kg a.s./ha for radish.

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The lowest 21-day ER_{50} values of glyphosate acid were observed in tomato plants and were calculated to be 0.5156, 0.3362 and 0.1457 kg glyphosate acid/ha for survival, plant height and dry weight, respectively. The lowest 21-day NOER values were determined to be 0.039 kg glyphosate acid/ha (tomato), 0.0392 kg glyphosate acid/ha (tomato and radish), and 0.314 kg glyphosate acid/ha (radish and tomato), respectively for plant height, survival, dry weight.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Glyphosate acid (N-phosphonomethylglycine)
Description:	White powder
Lot/Batch #:	RUD-9302-4778-T (technical) RUD-9203-3961-A (analytical standard)
Purity:	96.6% (technical) 99.8% (analytical standard)
2. Vehicle and/or positive control:	Deionized water containing a non-ionic surfactant
3. Test organism:	
Species:	6 Dicotyledons: (soybean[1], lettuce[2], cabbage[3], cucumber[4] radish[3] tomato[3]) 4 Monocotyledons: (oat[5], Ryegrass[6], corn [3],onion[3])
Source:	1. ███ 2. ███ 3. ███ 4. ███ 5. ███ 6. ███
4. Environmental conditions:	
Temperature:	19°C – 44°C(base test) 17°C – 40 (test continuation)
Relative humidity:	40% - 90%(base test) 37% - 90(test continuation)
Photoperiod:	Approx. 14 h light/ 10 h dark at. 38212– 45639 Lux (base test) Approx. 13 h light/ 11 h dark 24541– 19052 Lux (test continuation)
Soil pH:	7.9
Soil organic matter content:	1.1%

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B. STUDY DESIGN AND METHODS

1. Experimental treatments: Prior to treatment, seedlings were grown in plastic pots (approx.10 cm x 10 cm x 7.6 cm) completely filled with soil/perlite mixture. Soybean, cucumber, oat and corn were planted at a depth of 2.5 cm while the remaining six crops were planted at a depth of 1.3 cm. Each treatment/crop combination was replicated four times. Prior to treatment, seedlings were grown to 1-3 true leaves and then thinned to five plants of uniform height per pot. Glyphosate was diluted in water containing two drops of ███ surfactant per 250 mL of water (to represent a realistic spray concentration of surfactant) and applied once with an overhead flat-fan nozzle at approximately 378 L/ha (40 gallons/acre).The plants were treated with seven nominal concentrations, encompassing 0.0785, 0.1569, 0.3138, 0.6276, 1.2329,

2.5778 and 5.0436 kg a.s./ha. In addition, one negative control group (treated with deionized water) was tested. All applications of glyphosate were performed indoors with a spray booth equipped with a single Teejet 8001-E nozzle and a compressed air cylinder. After treatment plants were kept in a greenhouse at 17 – 44°C and 37 – 90% relative humidity under natural light supplemented with artificial lighting for 18 h per day. During the first 48 hours after treatment, pots were hand watered to prevent the test item from being washed off. As a no-observable effect concentration level was not reached for radish and tomato, a test continuation was initiated for both species using six nominal concentrations, encompassing 0.0049, 0.0099, 0.0202, 0.0392 and 0.0785 kg a.s./ha.

2. Observations: Plant height was recorded prior to treatment and 21 days after treatment. Phytotoxicity ratings were recorded 7, 14, and 21 days after treatment. 21 days after treatment, surviving plants were cut at soil level and dry weight was recorded. Prior to application, samples (10 mL) of each test solution were collected and analysed immediately by HPLC method to verify the concentrations of the test item in the test solutions.

3. Statistical calculations: Analysis of variance, followed by a one-tailed Dunnett's multiple comparison test were used for data analysis. The ER_x values were determined using regression analysis (TableCurve™ Curve Fitting Software).

II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

Visual phytotoxicity, plant height and plant dry weight of all crops were significant affected by glyphosate treatments. Except for soybean and onion, a significant effect on mortality was observed for all species exposed to glyphosate. The resulting ER_{50} and NOER values are presented in the table below.

Crop	Endpoint (kg a.s./ha) at Day 21					
	Survival		Plant height		Plant dry weight	
	NOER	ER_{50}	NOER	ER50	NOER	ER_{50}
Ryegrass	1.232	4.592	0.627	2.352	0.627	1.344
Corn	0.627	1.680	0.627	0.918	0.627	0.750
Onion	5.040	> 5.040	0.627	> 5.040	0.627	1.792
Oat	2.576	> 5.040	0.627	1.344	0.157	0.874
Soybean	5.040	> 5.040	0.627	1.568	0.314	0.974
Lettuce	1.232	2.800	0.627	1.344	0.314	0.762
Cucumber	2.576	4.032	0.314	1.456	0.314	0.896
Cabbage	1.232	4.592	0.627	1.456	0.157	0.739
Radish	0.314	0.918	0.078	0.358	0.039	0.246
Tomato	0.314	0.515	0.039	0.336	0.039	0.146

Analytical results: The average recovery of glyphosate in test media ranged from 100% to 107% and 105% to 110% of the nominal test concentrations for the first test and the test extension, respectively.

As the mean measured content of the test item always ranged between 80 and 120% of nominal in both tests, ecotoxicological endpoints were evaluated using nominal concentrations of the test item.

The validity criteria according to the OECD 227 were fulfilled.

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III. CONCLUSION

The lowest (worst case)21 day ER_{50} values of glyphosate acid were observed for tomato plants and were calculated to be 0.5156, 0.3362 and 0.1457kg a.s./ha for survival, plant height and dry weight, respectively. The lowest 21-day NOER values were determined to be 0.0785 kg a.s./ha (tomato and radish), 0.3138 kg a.s./ha (tomato and radish), 0.0392 kg a.s./ha (tomato and radish), and 0.0392 kg a.s./ha (tomato) respectively for visual phytotoxicity, survival, dry weight and plant height.

Annex point	Author(s)	Year	Study title
IIA 8.12/03	[redacted]	1996	Glyphosate acid: A Tier 2 greenhouse Study to Assess the Effects on Vegetative Vigour of Terrestrial Non-Target Plants. [redacted] Report No: [redacted] 2009B Date: 1996-09-16 GLP: yes Not published

Guideline: EPA Guidelines, Subdivision J, Series 123-1 (a)

Deviations: None

Dates of experimental work: Not stated

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Executive Summary

A vegetative vigour study was conducted exposing seven dicotyledonous (sugar beet, lettuce, radish, oilseed rape, cucumber, soybean and okra) and four monocotyledonous (purple nutsedge, oat, winter wheat and maize) plant species to six nominal test concentrations of glyphosate, encompassing 0.00615, 0.0184, 0.0553, 0.166, 0.498 and 1.49 kg a.s./ha. In addition, two negative control groups (untreated plants) were tested. Each test concentration was applied in three replicates containing five plants. Due to a local aphid infestation during the above-described test (test 1), a second test (test 2) was carried out for radish and oilseed rape only.

Following the application, plant damage and phytotoxic effects were recorded 6, 13, 21 and 27 days after application (DAA) in test 1 and 7, 14 and 28 DAA in test 2. For plant dry weight estimation, plants were harvested at 28 DAA by cutting the stem at soil level. All five plants for each replicate (3 samples per treatment and each control) were pooled together for drying.

With exception of purple nutsedge all species were severely affected (60 – 100%) at the two highest dose rates at the end of the study. Purple nutsedge was affected only at the highest treatment rate. The dry weight of all species except purple nutsedge was significantly affected ($p \leq 0.05$) at least at the two highest test item treatment rates. Purple nutsedge was affected only at the highest rate. Weight reduction of winter wheat, lettuce, oilseed rape and okra were significantly affected at the three highest treatment rates and for soybean at the highest four rates. Dry weight of oats was significantly reduced at all rates, except 0.0553 kg a.s./ha.

The validity of the present study according to OECD guideline 227 is questionable, as no data on seedling emergence and plant survival in control group were reported.

The worst case ER_{50} values of glyphosate were observed for oilseed rape and were calculated to be 0.140 kg glyphosate acid/ha and 0.150 kg glyphosate acid/ha, respectively for visual damage assessment and plant dry weight.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Glyphosate acid (formulated product)
Description:	Wettable powder (WP) formulation
Lot/Batch #:	F12
Purity:	50% a.s (nominal), 48.3 % a.s. (measured)
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	7 Dicotyledons: (sugarbeet[4], lettuce[5], radish[6], oilseed rape[1], cucumber[6], soybean[7], okra[6]) 4 Monocotyledons: (purple nutsedge[1], oat[2], winter wheat[1], , maize[3])
Source:	1. [redacted] 2. [redacted] 3. [redacted] USA; 4. [redacted] UK, [redacted] 5. [redacted] 6. [redacted]: 7. [redacted] [redacted] USA.
4. Environmental conditions:	
Temperature:	Test 1: 10-42 °C: Oats, winter wheat, sugar beet, lettuce, radish, and oilseed rape (cool); 9 – 45.5 °C: purple nutsedge, maize, cucumber, soybean and okra (warm) Test 2: 8.0 – 27.0 °C: radish, and oilseed rape (cool);
Relative humidity:	Test 1: 6 – 86% (cool), 3 – 100% (warm) Test 2: 24 – 80% (cool)
Photoperiod:	16 hours light / 8 hours dark Test 1: 80 – 651 klux (cool), 77 – 606 klux (warm) Test 2: 67 – 199 klux (cool)
Soil pH:	5.4 – 6.4 (test 1); 6.7 (test 2)
Soil organic matter content:	1.0 - 1.1% (test 1), 1.0 (test 2)

B. STUDY DESIGN AND METHODS

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1. Experimental treatments: Prior to treatment, seedlings were grown (in 10 cm- diameter plastic pots containing 9 cm depth of compost) to the 1-3 true leaf stage from untreated seed in SC compost (1.0 - 1.1% organic matter, pH 5.4 – 6.7) in a glasshouse. Applications of the formulated product were made using a hydraulic track sprayer fitted with a single even brass 8002 EVB Teejet. The plants were treated with six concentrations, 0.00615, 0.0184, 0.0553, 0.166, 0.498 and 1.49 kg a.s./ha. In addition, two negative control groups (untreated plants) were tested. Each test concentration was applied in three replicates containing five plants. After spraying, plants were left to dry for at least an hour before being transferred to a glasshouse and watered. Due to a localised aphid infestation of radish and oilseed rape during the test (test 1), a second test (test2) was carried out.

2. Observations: Following the application, plant damage and visual phytotoxicity was observed 6, 13, 21 and 27 days after application (DAA) in test 1 and 7, 14, and 28 DAA in test 2. Each plant was observed individually and compared to a representative healthy control plant of the same replicate and species. Assessments were carried out using a percentage damage scale.

For plant dry weight, plants were harvested at 28 DAA by cutting the stem at soil level. For each replicate, all plants were pooled together. Dry weight was assessed as g/replicate for each species by drying from the day of harvest until a constant weight was observed.

3. Statistical calculations: Dose response relationship for dry weight was estimated by non-linear logistic regression. The ER_x values were calculated as reduction from the observed control mean and Fieller's Theorem was used calculate their respective 95% confidence limits.

II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

Damage assessment: All species except purple nutsedge were severely affected (60 – 100%) at the two highest treatment rates. purple nutsedge was affected at the highest rate only. Predominant visual phytotoxicity symptoms were overall stunt, leaf necrosis, leaf chlorosis and senescence.

Plant dry weight: The dry weight of all species except purple nutsedge was significantly affected (α = 0.05) at the two highest test rates. Purple nutsedge was affected only at the highest test item treatment rate. Weight of winter wheat, lettuce, oilseed rape and okra was also significantly reduced at the third highest test item treatment rate (0.166 kg a.s./ha) and for soybean at the test item treatment rate of 0.0553 kg a.s./ha. Dry weight of winter oats was significantly reduced at all rates tested, except at 0.0553 kg a.s./ha.

Crop	Endpoint [kg a.s./ha]			
	Damage assessment		Plant dry weight	
	ER_{25}	ER_{50}	ER_{25}	ER_{50}
Purple nutsedge (*Cyperus rotundus*)	0.891	1.293	0.851	1.253
Oat (*Avena sativa*)	0.274	0.415	0.281	0.376
Winter wheat (*Triticum aestivum*)	0.270	0.390	0.178	0.242
Maize (*Zea mays*)	0.268	0.386	0.397	0.423
Sugar beet (*Beta vulgaris*)	0.254	0.416	0.199	0.377
Lettuce (*Lactuca sativa*)	0.307	0.466	0.254	0.402
Radish (*Raphanus sativus*)	0.228	0.417	0.488	1.078
Oilseed rape (*Brassica napus*)	0.083	0.149	0.106	0.150
Cucumber (*Cucumis sativa*)	0.138	0.254	0.205	0.359
Soybean (*Glycine max)*	0.190	0.415	0.174	0.358

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Okra (***Abelmoschus esculentus***)	0.256	0.507	0.205	0.346

The validity of the present study according to OECD guideline 227 is questionable, as no data on seedling emergence and plant survival in control group were reported.

III. CONCLUSION

The lowest ER_{50} values of glyphosate acid were observed with oilseed rape and were calculated to be 0.140 kg a.s./ha and 0.150 kg a.s/ha, respectively for damage assessment and plant dry weight.

Annex point	Author(s)	Year	Study title
IIA 8.12/04	[redacted]	1996	Glyphosate acid: A Tier 2 greenhouse Study to Assess the Effects on Seedling Emergence of Terrestrial Non-Target Plants. [redacted] Report No: [redacted] 2008B Date: 1996-09-19 GLP: yes Not published

Guideline: EPA Guidelines, Subdivision J, Series 123-1 (a)

Deviations: None

Dates of experimental work: Not stated

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Executive Summary

A seedling emergence study was conducted exposing seven dicotyledonous (sugar beet, lettuce, oilseed rape, cucumber, soybean, okra and rhubarb) and five monocotyledonous (purple nutsedge, oat, winter wheat, maize, and onion) plant species to six nominal test concentrations of glyphosate, encompassing 0.140, 0.280, 0.560, 1.12, 2.24 and 4.48 kg a.s./ha. In addition, two negative control groups (untreated test trays) were tested. For each of the twelve species, three replicates trays (containing each 10 seeds) were prepared for each of the six treatment rates and two controls.

Because of high control mortality of okra, soybean and lettuce caused by extreme temperature conditions during test 1, a second test (test2) was carried out for these species.

Following emergence, plant development was observed 14 and 27 days after application (DAA) in test 1 and 14 and 28 DAA in test 2. For dry weight assessment, the plants were harvested at 28 DAA.

Results showed no effects of glyphosate acid on seedling emerge when compared to the control. For visual damage, differences (generally stem decrease) from the controls were evident for all species. However, there was no consistent pattern or dose response. Therefore these effects were not considered treatment-related. For dry weight, no significant dose-related reduction was observed. For purple nutsedge, three dose rates (0.280, 0.560 and 2.24 kg a.s./ha) resulted in significant differences when compared to the controls. The highest, third highest and lowest rates were however not significantly different from the controls and therefore the endpoint could not be determined.

The validity of the present study according to OECD guideline 208 is questionable, as no data on visible phytotoxic effects and mean survival of emerged seedlings in control group were reported.

Under the conditions of the present study, theER_{50} and NOER of glyphosate acid were determined to be > 4.48 kg a.s/ha and 4.48 kg a.s/ha respectively for both seedling emergence and seedling dry weight, except for the dry weight of purple nutsedge, where no endpoints could be determined.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	Glyphosate acid (formulated product)
Description:	Wettable powder (WP) formulation
Lot/Batch #:	F12
Purity:	50% a.s (nominal), 48.3 % a.s. (measured)
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	7 Dicotyledons: (sugar beet [5], lettuce [6], oilseed rape [1], cucumber [4], soybean [7], okra [4] and rhubarb [4]) 5 Monocotyledons: (purple nutsedge [1], oat [2], winter wheat [1], maize [3], and onion [4])
Source:	1. 2. 3. USA; 4. 5. 6. 7. USA.
4. Environmental conditions:	
Temperature:	Test 1: 10-42 °C: Oats, winter wheat, sugar beet, lettuce, radish, oilseed rape, onion and rhubarb (cool); 9 – 45.5 °C: purple nutsedge, maize, cucumber, soybean and okra (warm) Test 2: 12.0 – 33.5 °C (cool); 13.0 – 39.5 °C (warm);
Relative humidity:	Test 1: 6 – 90% (cool), 3 – 100% (warm) Test 2: 12 – 94% (cool), 4 – 94% (warm)
Photoperiod:	16 hours light / 8 hours dark Test 1: 96 – 6483 klux (cool), 71 – 669 klux (warm) Test 2: 68 – 394 klux (cool); 56 – 524 klux (warm);
Soil pH:	6.4 (test 1); 6.5 (test 2)
Soil organic matter content:	1.0 (test 1), 0.7 (test 2)

B. STUDY DESIGN AND METHODS

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1. Experimental treatments: Ten seeds per replicate per species were sown into disposable plastic seed trays (11 cm x 15 cm x 7 cm, with base holes) containing equal-sized compartments and filled to top (7 cm depth) with SC compost (0.7 – 1.0% organic matter), For each of the twelve species, three replicate trays were prepared for each treatment rate and two controls. After one day, seed trays were treated with 6 nominal test concentrations, 0.140, 0.280, 0.560, 1.12, 2.24 and 4.48 kg a.s./ha. In addition, two negative control groups (untreated test trays) were tested. Each test concentration was applied using a hydraulic track sprayer fitted with a single, even brass 8002 EVB Teejet. After spraying, the trays were left to dry for 3 hours before being transferred to a glasshouse and watered. Because of a high control mortality for okra, soybean and lettuce during the first test (test 1) caused by extreme temperature conditions, a second test (test2) was carried out with these species.

2. Observations: Emergence: Seedling emergence was assessed three times a week by recording the number of seeds emerged per replicate.

Visual damage: Plant development was observed 14 and 27 days after application (DAA) in test 1 and 14 and 28 DAA in test 2. Each replicate was assigned a single overall score at each assessment. Non-emergence was included in the overall rating as a percentage of decrease compared to controls. Observation of phytotoxic symptoms was also recorded. Control replicates were given a zero score and treated replicate were assessed relative to the control.

Dry weight: The plants were harvested at 28 DAA by cutting the stem at soil level. Dry weight was assessed as g/ replicate for each species by drying in an oven at approximately 70°C starting at the day of harvest until a constant weight was observed.

3. Statistical calculations: Data were analysed using a t-test ($\alpha = 0.05$), after an arcsine transformation of the data (for emergence only). No discernible dose response was detected and therefore the estimation of ERx values was not considered for both emergence and dry weight.

II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

Emergence: Seedling emergence was unaffected by the application of glyphosate acid for all species, when compared to the controls.

Visual damage and phytotoxicity: Visual differences from the controls were evident with all species, this being generally attributable to stem decrease. However, there was no consistent dose response pattern and therefore effects were not considered be treatment-related. Mortality was observed at treated lettuce and okra plants. This mortality was not considered to be treatment-related as it generally occurred after full emergence.

Dry weight: there was no significant dose-related reduction of mean dry weight. For purple nutsedge, three dose concentrations resulted in significant differences from the controls (0.280, 0.560 and 2.24 kg a.s./ha). The highest, third highest and lowest rates were however not significantly different from the controls and therefore no endpoint could be determined.

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Crop	Endpoint [kg a.s./ha]			
	Emergence		Dry weight	
	ER_{50}	NOER	ER_{50}	NOER
Purple nutsedge (*Cyperus rotundus*)	> 4.48	4.48	nd	nd
Oat (*Avena sativa*)	> 4.48	4.48	> 4.48	4.48
Winter wheat (*Triticum aestivum*)	> 4.48	4.48	> 4.48	4.48

Maize (*Zea mays*)	> 4.48	4.48	> 4.48	4.48
Onion (*Allium cepa*)	> 4.48	4.48	> 4.48	4.48
Sugar beet (*Beta vulgaris*)	> 4.48	4.48	> 4.48	4.48
Lettuce (*Lactuca sativa*)	> 4.48	4.48	> 4.48	4.48
Oilseed rape (*Brassica napus*)	> 4.48	4.48	> 4.48	4.48
Cucumber (*Cucumis sativa*)	> 4.48	4.48	> 4.48	4.48
Soybean (*Glycine max)*	> 4.48	4.48	> 4.48	4.48
Okra (*Abelmoschus esculentus*)	> 4.48	4.48	> 4.48	4.48
Rhubarb (*Rheum rhoponticum*)	> 4.48	4.48	> 4.48	4.48

The validity of the present study according to OECD guideline 208 is questionable, as no data on visible phytotoxic effects and the mean survival of emerged seedlings for the duration of the study in control group were reported.

III. CONCLUSION

The$ER_{50}$and NOER of glyphosate acid were determined to be > 4.48 kg a.s/ha and 4.48kg a.s/ha respectively for both seedling emergence and seedling dry weight of oat, winter wheat, maize, onion, sugar beet, lettuce, oilseed rape, cucumber, soybean, okra and rhubarb.

Annex point	Author(s)	Year	Study title
IIA 8.12/05	[redacted]	2005	Evaluation of the toxicity of Glyphosate and Paraquat to terrestrial non-target plants [redacted] Report No: [redacted].104 Date: 2005-09-19 GLP: no Not published

Guideline: OECD Guideline 208B (draft, 2000)

Deviations to OECD 227: No data on phytotoxicity available, control mortality was not reported, data on seedling emergence for garden cress and winter wheat not available. No chemical analytics were conducted.

Dates of experimental work: July 2001

Executive Summary

A Tier 2 vegetative vigour study using MON 52276 (active substance: glyphosate) and [redacted] (active substance: paraquat) was conducted exposing four dicotyledonous (sugar beet, rape, garden cress, pea) and two monocotyledonous species (ryegrass and wheat) to seven nominal test concentrations of MON52276, encompassing 0.00004 to 4.0 L prod/ha, equivalent to 0.000014 to 1.44 kg a.e./ha in four replicates per treatment. In addition, a negative control group treated with deionized water was tested. The test material was applied to plants at least at 2 to 4 leaf stage using a pressurized pot sprayer.

Visual assessments were made for signs of phytotoxicity immediately before spraying, at days 1, 2, 3, 4, 7, 15 and 22 after treatment. Fresh shoot weight was determined 22 days after treatment.

Among dicotyledonous species, sugar beet (*Beta vulgaris*) and rape (*Raphanus rapistrum*) exhibited significant effects one day after application at an application rate of 720 g a.e./ha. Garden cress (*Lepidum sativum*) was the most sensitive species from day 2 until the end of the study ($NOER_{phytotox}$ =

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14.4 g a.e./ha). Among monocotyledonous species, perennial ryegrass was more tolerant to MON 52276 than winter wheat ($NOER_{phytotox}$ = 14.4 g a.e./ha). Since there was a 10-fold separation factor between levels at the lower end of the dose response curves, these NOEC values are likely very conservative. ER_{50} values were calculated for six test species, including sugar beet, rape, garden cress, pea, ryegrass, and winter wheat, and ranged from 0.252 kg a.e./ha for garden cress to 0.597 kg a.e./ha for pea. The validity criteria according to the OECD 227 could not be assessed, since no data on phytotoxic effects or mean plant survival in the control are available.

ER_{50} values for non-target plants exposed to MON 52276 ranged from 0.252 kg a.e./ha for garden cress to 0.597 kg a.e./ha for pea. The lowest NOER values of 14.4 g a.e./ha (equivalent to 0.04 L prod/ha) for MON52276 were observed for garden cress and winter wheat for phytotoxicity. The NOER based on plant fresh weight was 144 g a.e./ha for all crops.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item::	MON 52276 (360 g a.s./L EC)
Description:	Liquid
Lot/Batch #:	not available
Purity:	not stated
2. Vehicle and/or positive control:	none
3. Test organism:	
Species:	4 Dicotyledons: (sugar beet, rape, garden cress, pea) 2 Monocotyledons: (perennial ryegrass, winter wheat)
Source:	Herbiseed ([redacted])
4. Environmental conditions:	
Temperature:	18 °C/12 °C day/night
Relative humidity:	not specified, substrates were watered daily
Photoperiod:	14 h light/ 10 h dark
Soil pH:	No data
Soil organic matter content:	No data

B. STUDY DESIGN AND METHODS

1. Experimental treatments: Prior to treatment, seedlings were grown in pots filled with sterilised Kettering loam and Derby Quartz (mixture loam and grit: 5:1). For sugar beet, rape, garden cress and pea, 6 seeds and for ryegrass and winter wheat, 12 seeds were planted per pot at a depth of 1 cm. Once a day or as required to ensure the soil surface remains moist, pots were watered with an automatic overhead boom pre-spray. Following seedling emergence, plants were thinned by removing half of the seedlings. Each treatment/crop combination was replicated four times. Prior to treatment, seedlings were grown to at least 2-4 true leaves. MON 52276 was applied indoors with a Mardrive pot sprayer at 225 L/ha at a height of 50 cm and at a pressure of 2.2 bar. The plants were treated with seven nominal concentrations, encompassing 0.00004, 0.0004, 0.004, 0.04, 0.4, 2.0 and 4.0 L prod/ha. In addition, one negative control group was tested. After treatment plants were kept in a greenhouse at 12 – 18°C h per day.

2. Observations: Phytotoxicity ratings, according to a nine point scoring system were recorded for the first 4 days and at approximately 7, 15 and 22 days after treatment. All plots were harvested 22 days after treatment to determine fresh shoot weight. The weight of plants in one pot was combined.

3. Statistical calculations: Data for the No Observed Effect Rates (NOER) were analysed using one-way ANOVA and Dunnett's t-test was performed as post-hoc. The highest concentration not significantly different from the control was identified as the NOER. ER_{50} values were calculated in a different report (Levine, S.L. & Orr, T.B., 2011, MSL0024009 IIIA 17.1/01). EC_{50} values and 95% confidence intervals were calculated from mean fresh shoot weights for each glyphosate treatment obtained 20 to 22 days after post-emergent application, using a standard 3-parameter logistic model with the software package GraphPad Prism version 5.04 ([redacted]).

II. RESULTS AND DISCUSSION

A. FINDINGS

The endpoints are based on nominal concentrations, as no analytical data are available. The resulting ER_{50} and NOER values based on biomass measured as fresh shoot weight are presented in the table below.

Crop	Endpoint (kg a.e./ha) at Day 21	
	Biomass	
	ER_{50} (95% C.L.)	NOER
Sugar beet	0.307 (0.187 – 0.427)	0.144
Rape	0.511 (0.0907 – 0.930)	0.144
Garden Cress	0.252 (0.124 – 0.380)	0.144
Pea	0.597 (0.408 – 0.785)	0.144
Perennial ryegrass	0.507 (0.144 – 0.72)	0.144
Winter wheat	0.344 (0.144 – 0.72)	0.144

B. OBSERVATIONS

Among dicotyledonous species, sugar beet (*Beta vulgaris*) and rape (*Raphanus rapistrum*) exhibited significant effects on vegetative vigour one day after application at an application rate of 720 g a.e./ha (equivalent to 2.0 L/ha). Garden cress (*Lepidum sativum*) was the most sensitive species from day 2 until the end of the study (NOER = 0.04 L/ha). Among monocotyledonous species, perennial ryegrass was more tolerant to MON 52276 than winter wheat (NOER = 0.04 L/ha, equivalent to 14.4 g a.e./ha). The NOEC for fresh shoot weight was the same for all species tested (NOER = 0.4 L/ha, equivalent to 144 g a.e./ha). Since there was a 10-fold separation factor between levels at the lower end of the dose responses curves, these NOEC values are likely very conservative.

Table 8.12-4: NOER of MON 52276 to monocotyledonous and dicotyledonous plants based on phytotoxicity and fresh shoot weight.

Crop	Phytotoxicity [L/ha]							Fresh shoot weight [L/ha]
	1 day	2 days	3 days	4 days	7 days	15 days	22 days	
Sugar beet	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Rape	0.4	2.0	0.4	0.4	0.4	0.4	0.4	0.4
Garden Cress	n.d.	0.04	0.04	0.04	0.04	0.04	0.04	0.4
Pea	n.d.	n.d.	0.4	0.4	0.4	0.4	0.4	0.4
Perennial ryegrass	n.d.	0.4	0.04	0.4	0.4	0.4	0.4	0.4
Winter wheat	n.d.	0.4	0.4	0.4	0.4	0.4	0.04*	0.4

n.d.: No significant differences between any treatment and the control
* 0-00004 L prod/ha also differed significantly from the control on this one sampling occasion

The validity criteria according to the OECD 227 could not be assessed, since no data on phytotoxic effects or mean plant survival in the control are available. The seedling emergence was > 70% in sugar beets, rape, pea and winter wheat. No data for garden cress and perennial ryegrass were reported.

III. CONCLUSION

ER_{50} values for non-target plants exposed to MON 52276 ranged from 0.252 kg a.e./ha for garden cress to 0.597 kg a.e./ha for pea. The lowest NOER values of 14.4 g a.e./ha (equivalent to 0.04 L prod/ha) for MON52276 were observed for garden cress and winter wheat for phytotoxicity. The NOER based on plant fresh weight was 144 g a.e./ha for all crops. Since there was a 10-fold separation factor between levels at the lower end of the dose responses curves, these NOEC values are likely very conservative.

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IIA 8.13 Effects on terrestrial vertebrates other than birds/wild mammal toxicity

Tests on other non-target species are not required by Directive 91/414/EEC and Regulation 1107/2009.

IIA 8.14 Effects on other non-target organisms (flora and fauna) believed to be at risk

See IIA 8.9.

IIA 8.14.1 Summary of all available data from preliminary tests used to assess biological activity and dose range finding which may provide information on other non-target species (flora and fauna)

The data presented above are considered sufficient to characterise the active substance glyphosate acid and its metabolites concerning the risk to non-target organism. Further preliminary tests are not deemed necessary in addition to the reported studies on representative species compiled in this dossier.

IIA 8.14.2 A critical assessment as to the relevance of the preliminary test data to potential impact on non-target species

See IIA 8.14.1.

IIA 8.15 Effects on biological methods of sewage treatment

At the time of the previous Annex I inclusion, no evaluation of effects on biological sewage treatment was required. Therefore, no studies are presented within the scope of Commission Document 6511/VI/99-final dated 21 January 2002.

A summary of the endpoints of the most sensitive species is presented in Table 8.15-1 whilst full details of these studies are provided below.

Table 8.15-1: Effects of glyphosate and formulation MON 52276 on biological methods of sewage treatment

Test substance Test type	Test organism	Endpoint [mg/L]	Reference/GLP
Glyphosate acid	*Pseudomonas putida*	16 h IC_{50} >100	IIA 8.15/01 █ 6889/B █ 2000/yes
Glyphosate acid	Activated sludge (domestic)	3 h EC_{50} >100	IIA 8.15/02 277830 █ 1990/yes

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Annex point	Author(s)	Year	Study title
IIA 8.15/01		2000	Glyphosate technical: Determination of toxicity to *Pseudomonas putida* Report No: 6889/B Date: 2000-06-19 GLP: yes not published

Guideline: Water quality - *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test) International Standard ISO 10712: 1995.

Deviations to ISO 10712: None

Dates of experimental work: 2000-05-11 to 2000-05-18

Executive Summary

The effects of glyphosate acid on *Pseudomonas putida* growth inhibition were evaluated in a 16-hour static toxicity test. The test concentrations of 1.0, 3.2, 10, 32, and 100 mg a.s./L in test medium were prepared in duplicate and sterile conditions in conical flasks. Flasks containing 1.0, 3.2, 10, 32, and 100 mg a.s./L (single replicates) of the reference toxic substance (3,5-dichlorophenol) and three control flasks were also prepared. Four mL growth medium, 1 mL inoculum and dejonised water were added to obtain a final volume of 50 mL test solution. After shaking at 27.0±0.5°C (in an in incubator) for 16±1 hours the optical density of the contents of each flask were measured with a spectrophotometer. All validity criteria according to the guideline ISO 10712 were fulfilled.

The 16-h IC_{50} for *Pseudomonas putida* exposed to glyphosate was >100 mg/L based on nominal concentration. The NOEC after 16 h was 100 mg test item/L.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item: Glyphosate acid
Aspect: White solid
Lot/Batch #: P30
Purity: 97.6%

2. Vehicle and/or positive control: 3,5-dichlorophenol (97%)
Growth medium

3. Test organism:

Species: *Pseudomonas putida*, strain NCIMB9494
Source of organisms:

UK

4. Environmental conditions:

Temperature: 27.0±0.5°C

B: STUDY DESIGN AND METHODS

1. Experimental treatments: The effects of glyphosate acid on *Pseudomonas putida* growth inhibition were evaluated in a 16-hour static toxicity test. The test concentrations of 1.0, 3.2, 10, 32, and 100 mg a.s./L in test medium were prepared in duplicate and sterile conditions in conical flasks. These test solutions were prepared by adding the appropriate amount of a 500 mg/a.s./L stock solution (0.125 g glyphosate acid in 250 mL deionised water) directly into the flasks. Flasks containing 1.0, 3.2, 10, 32, and 100 mg a.s./L (single replicates) of the reference toxic substance (3,5-dichlorophenol) and three control flasks were also prepared. Four mL growth medium, 1 mL inoculum and deionised water were added to obtain a final volume of 50 mL test solution. After shaking at 27.0±0.5°C (in an in incubator) at 150 rpm for 16±1 hours the optical density of the contents of each flask were measured at 600 nm with an Uvikon 930 spectrophotometer.

II. RESULTS AND DISCUSSION

A: FINDINGS AND OBSERVATIONS

The effects of glyphosate acid on *Pseudomonas putida* are shown below.

Table 8.15-2: Effects of glyphosate acid on *Pseudomonas putida*

Nominal concentration [mg a.s./L]	Mean optical density	Mean % inhibition
Control	0.859	-0
1.0	0.836	3
3.2	0.838	2
10	0.842	2
32	0.868	0
100	0.878	0
3,5-DCP 1.0	0.839	2
3,5-DCP 3.2	0.857	0
3,5-DCP 10	0.851	1
3,5-DCP 32	0.055	94
3,5-DCP 100	0.047	95

All validity criteria according to ISO 10712were fulfilled, as control inoculum has multiplied by a factor of at least 60 within the test period and the EC_{50} of the reference substance 3,5-dichlorophenol was between 10 mg/L and 30 mg/L.

III. CONCLUSION

The 16-h IC_{50} for *Pseudomonas putida* exposed to glyphosate acid was >100 mg/L based on nominal concentration. The NOEC after 16 h was 100 mg test item/L.

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Annex point	Author(s)	Year	Study title
IIA 8.15/02		1990	Assessment of the acute toxicity of glyphosate technical on aerobic waste-water bacteria Report No: 277830 Date: 1990-10-17 GLP: yes not published

Guideline:	OECD 309
Deviations:	None
Dates of experimental work:	1990-07-19 (start and end)

Executive Summary: The effects of glyphosate acid on activated sludge were determined in a 3-hour exposure laboratory study. Activated sludge from a domestic waste-water treatment plant was exposed to the test item at concentrations of 3.2, 10, 32, 50, and 100 mg a.s./L, 2 untreated controls and a toxic reference (3,5-dichlorophenol at concentrations of 1.0, 3.2, 10, 32, and 50 mg a.s./L). After 180 minutes of aeration at 22°C, samples were taken from the test flasks for oxygen measurement over a period of up to 10 minutes. The inhibitory effect of the test item is expressed as oxygen consumption per minute. All validity criteria according to the guideline OECD 209 were fulfilled.

The EC_{50} for waste-water micro-organisms exposed to glyphosate acid was determined to be >100 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate acid
Aspect:	White solid
Lot/Batch #:	229-Jak-5-1
Purity:	98.9%

2. Vehicle and/or positive control: 3,5-dichlorophenol (97%)

3. Test organism:

Source of activated sludge: Switzerland

4. Environmental conditions:

Temperature:	22°C
pH:	7.5-7.7

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B: STUDY DESIGN AND METHODS

1. Experimental treatments: The effects of glyphosate acid on activated sludge were determined in a 3-hour exposure laboratory study. Activated sludge from a domestic waste-water treatment plant was exposed to the test item at concentrations of 3.2, 10, 32, 50, and 100 mg a.s./L, 2 untreated controls and a toxic reference (3,5-dichlorophenol at concentrations of 1.0, 3.2, 10, 32, 50, and 100 mg a.s./L). A stock solution of 500 mg a.s./L was prepared by dissolving glyphosate acid in bi-distilled water. The sludge was sieved, centrifuged and the solid material re-suspended in tap water and again centrifuged. This procedure was repeated a further 2 times. An aliquot of the final sludge suspension was made up with Soerensen buffer to 1 litre. To that mixture, 50 mL OECD recommended synthetic sewage feed were added.

Glass flasks were filled with appropriate aliquots of stock solutions, water and activated sludge up to 500 mL final volume and aerated with an air flow of about 0.2 L/minute. After 180 minutes of aeration at 22°C, samples were taken from the test flasks for oxygen measurement over a period of up to 10 minutes. The inhibitory effect of the test item is expressed as oxygen consumption per minute.

2. Observations: Respiration rate (oxygen consumption expressed as mg O_2 per litre per hour) was expressed as percent inhibition relative to the control.

II. RESULTS AND DISCUSSION

A: FINDINGS

The influence of glyphosate acid on oxygen consumption of activate sludge is presented below.

Table 8.15-3: Influence of glyphosate acid on oxygen consumption of activate sludge

Nominal concentration [mg a.s./L]	Oxygen consumption [mg O_2 per litre per hour]	Mean [deviation]	Inhibition [%]
Control	1.02	1.085 (12.7%)	-
Control	1.15		-
3.2	1.16	-	-6.9
10	1.09	-	-0.5
32	1.15	-	-6.0
50	1.09	-	-0.5
100	1.17	-	-7.8
3,5-DCP 1.0	1.11	-	-2.3*
3,5-DCP 3.2	1.07	-	1.4*
3,5-DCP 10	0.38	-	65.0*
3,5-DCP 32	0.07	-	93.5*
3,5-DCP 50	0.05	-	95.4*

B. OBSERVATIONS

The table shows that the respiration rate of the sludge was not inhibited (-7.8%) at the highest concentration of glyphosate acid of 100 mg a.s./L. The EC_{50} for the toxic reference 3,5-DCP was found to be 8.6 mg/L.

All validity criteria according to OECD 209 were fulfilled, as oxygen uptake rates of blank controls were not less than 20 mg oxygen per 1 g of activated sludge, the coefficient of variance for oxygen uptake in

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the control replicates was not more than 30% and the EC_{50} of 3,5-DCP was in the range expected and specified in the pertaining guideline OECD 209.

III. CONCLUSION

The EC_{50} for waste-water micro-organisms exposed to glyphosate acid was determined to be>100 mg/L.

IIA 8.16 Other/special studies

IIA 8.16.1 Other/special studies - laboratory studies

Annex point	Author(s)	Year	Study title
IIA 8.16.1/01		2012	Comparative Post-Emergence Phytotoxicity of AMPA and Glyphosate to Crop and Annual Weed Species Report No.: 0024009 Date: 2012-04-10 GLP: no Not published

Guideline: None

Deviations: Not applicable

Dates of experimental work: 1986-03-12 and 1986-08-15

Executive Summary

EC_{50} values were calculated to compare relative post-emergence phytotoxicity between glyphosate and the glyphosate degradate aminomethylphosphonic acid (AMPA) with crop and annual weed species. Foliar applications of AMPA are unrealistic and were only performed with the intent of comparing the relative potency of AMPA and glyphosate. All phytotoxicity data were generated in green houses in herbicide screening studies. EC_{50} values for glyphosate acid and AMPA were compared on a molar basis to assess relative toxicity. EC_{50} molar ratios were calculated as EC_{50} AMPA/ EC_{50} glyphosate acid and ranged from 3.4 for hemp sesbania to 86.8 for common lambsquarters.

EC_{50} molar ratios were calculated as EC_{50} AMPA/ EC_{50} glyphosate acid and ranged from 3.4 for hemp sesbania (*Sesbania exaltata*) to 86.7 for common lambsquarters (*Chenopodium album*) with an average ratio across the seventeen tested species of 22, indicating that AMPA has significantly lower herbicidal activity compared to glyphosate.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Glyphosate isopropylamine (IPA) salt (N-phosphonomethylglycine) AMPA (Aminomethylphosphonic acid)
Description:	Glyphosate IPA: Viscous liquid AMPA: White powder
2. Vehicle and/or positive control:	Deionized water containing 0.4% emulsifier-L (cyclo-L)
3. Test organism:	
Species:	9 Dicotyledons: (cocklebur, hemp sesbania, lambsquarters, morning glory, smartweed, soybean, sugar beet, velvetleaf, wild buckwheat) 8 Monocotyledons: (barnyard grass, corn, crabgrass, green foxtail, proso millet, rice, sorghum, wheat)
Source:	Obtained from a supply of viable seeds kept in suitable storage facilities by █████ to maintain viability

B. STUDY DESIGN AND METHODS

1. Experimental treatments: At planting, containers were packed with a sufficient quantity of sterilized silt loam soil to provide a uniform medium for plant growth. Seeds were planted in sufficient quantity to give between 5 and approximately 30 specimens per replicate depending on the species planted and covered with a half-inch layer of soil. After planting, plants were moved to the greenhouse with supplementary lighting to support good plant growth and placed on a bench containing capillary matting. Sufficient tap water was provided to promote germination and provide good plant growth. Nominal test concentrations for foliar applications were prepared from a 1% stock solution for glyphosate acid equivalent and AMPA and applied as needed to achieve the desired rate of application to young plants (approximately two weeks after planting) using a spray bottle fitted with a DeVilbiss No. 152 atomizer nozzle. Low rates required further dilution of the 1% stock solution to 0.1% and 0.01% stock solutions to ensure accuracy in pipetting. To complete the formulation prior to application 0.4% of emulsifier-L (cyclo-L) was added to each spray bottle and then water was added in sufficient volume to provide a spray volume of 200 gallons/A. The spray solutions were uniformly applied to the foliage of the plants. The plants were inspected approximately twice per week by a trained observer and observations were recorded. Phytotoxicity was recorded as visual percent injury (chlorosis) relative to the untreated control and evaluated two weeks after test initiation.

2. Observations: Phytotoxicity was recorded as visual percent injury (chlorosis) relative to the untreated control and evaluated two weeks after test initiation. The percent injury observations were used as the phytotoxicity endpoint to calculate EC_{50} values in this analysis. For this linear and quantitative scale a value of 0 = 0% chlorosis, 10 = 10% chlorosis and so on.

3. Statistical calculations: EC_{50} values were calculated using a 3-parameter logistic model with the software package GraphPad Prism version 5.04 (█████). The maximum asymptote was constrained in the logistic model to 100% to reflect the maximum potential response based on percent injury observations.

II. RESULTS AND DISCUSSION

A. FINDINGS and OBSERVATIONS

EC_{50} values for glyphosate acid and AMPA were compared on a molar basis to assess relative toxicity. EC_{50} molar ratios were calculated as EC_{50} AMPA/ EC_{50} glyphosate acid and ranged from 3.4 for hemp sesbania to 86.8 for common lambsquarters (Table 1). The mean EC_{50} AMPA/ EC_{50} glyphosate acid ratio across the seventeen species was 22, indicating a significantly lower toxicity of AMPA compared to glyphosate (Table 8.16.1-1). In total, 12 of the 17 ratios are between the ratios of 11 and 30. In all cases, the ratios were greater than two, indicating that AMPA has less than 50% of the herbicidal activity of glyphosate.

Table 8.16.1-1: Post-emergence EC_{50} values for AMPA and Glyphosate to Crop and Annual Weed Species based on units of moles/ha

Species Common name	Species Scientific Name	Glyphosate Acid EC_{50} (Moles/ha)	AMPA EC_{50} (Moles/ha)	EC_{50} Molar Ratio [1]
BARNYARD GRASS	*Echinochloa crus-galli*	4.202	103.972	24.745
COCKLEBUR	*Xanthium strumarium*	4.123	25.963	6.297
CORN	*Zea mays*	1.710	42.346	24.762
CRABGRASS	*Digitaria ischaemum*	2.320	70.661	30.458
GREEN FOXTAIL	*Setaria veridis*	2.146	42.790	19.937
HEMP SESBANIA	*Sesbania exaltata*	5.933	20.159	3.398
LAMBSQUARTERS	*Chenopodium album*	2.303	199.869	86.773
MORNING GLORY	*Ipomoea sp.*	6.913	128.603	18.602
PROSO MILLET	*Panicum miliaceum*	1.949	43.668	22.401
RICE	*Oryza sativa*	5.537	87.650	15.831
SMARTWEED	*Polygonum pensylvanicum*	4.882	37.682	7.718
SORGHUM	*Sorghum bicolor*	3.310	97.663	29.504
SOYBEAN	*Glycine max*	5.618	92.677	16.496
SUGAR BEET	*Beta vulgaris*	3.291	39.237	11.923
VELVETLEAF	*Abutilon theophrasti*	5.204	142.432	27.370
WHEAT	*Triticum aestivum*	4.703	131.732	28.007
WILD BUCKWHEAT	*Polygonum convolvulus*	3.287	25.821	7.856
AVERAGE		**3.9667**	**78.407**	**22.475**

[1] EC_{50} ratio calculated as EC_{50} AMPA/ EC_{50} glyphosate. Results were calculated with full precision mode in Excel and may differ slightly from hand calculated values.

III. CONCLUSION

EC_{50} molar ratios were calculated as EC_{50} AMPA/ EC_{50} glyphosate acid and ranged from 3.4 for hemp sesbania to 86.7 for common lambsquarters. All AMPA/ EC_{50} glyphosate acid ratios were greater than 2, with an average ratio across the seventeen tested species of 22, indicating that AMPA has significantly lower (< 50%) herbicidal activity compared to glyphosate.

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IIA 8.16.2 Other/special studies - field studies

The following field study was performed to characterize residues of glyphosate on insects and as a potential refinement for the avian risk assessment.

Annex point	Author(s)	Year	Study title
IIA 8.16.2./01	[redacted]	2010	Residues of glyphosate in arthropods after spray application in an arable field – magnitude and time course of residue decline [redacted] Report No: 10153 Date: 2010-12-14 GLP: yes not published

Guideline: Guidance of EFSA: Risk Assessments for Birds and Mammals (2009)

Deviations: None

Dates of experimental work: 2010-04-14 to 2010-05-26

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Executive Summary

Field studies were undertaken to determine the residue levels of glyphosate in ground-dwelling arthropods in an arable field over 35 days after spray application of the test item MON 52276 (a.s. glyphosate acid). The aim of the test was:

- to determine the initial and maximum glyphosate concentration in ground-dwelling arthropods after spray application of 1 x 2.88 kg glyphosate acid equivalents/hectare and
- to determine the time course of decline of this residues during the sampling period to allow calculation of time-weighted average concentrations for different time windows.

The test item MON 52276 was applied once at a nominal application rate of 8 L formulation/ha in a spray volume of 200 L water /ha (corresponding to 2.88 kg a.s./ha). Samples of the natural population of ground-dwelling arthropods were collected for residue analysis by pitfall trapping. All glyphosate residues present in ground-dwelling arthropods collected from the study plots on the day of application of MON 52276 (DAT 0, before application) were below the LOD of the analytical method (<0.03 mg a.s./kg f.w.). On DAT 1, the mean initial concentration of glyphosate in sampled ground-dwelling arthropods was found to be 5.97 ± 0.78 mg a.s./kg f.w. The mean maximum concentration of glyphosate over the sampling period occurred on DAT 1. The maximum 90th percentile of glyphosate residues over the sampling period was measured on DAT 2 (7.30 mg a.s./kg f.w). The maximum-time weighted average (TWA) of a 21-day period (moving window) was 2.26 mg a.s./kg f.w. The TWA of glyphosate in ground dwelling arthropods for the whole sampling period (DAT 1-35) was found to be 1.64 mg a.s./kg f.w. The residues per unit dose (RUD) for the mean initial residue in ground dwelling arthropods was 2.08. RUD for the maximum 21-day TWA was 0.79.

In conclusion, the study provides realistic data on the magnitude of peak residue levels and the corresponding time course of residue decline of glyphosate in ground-dwelling arthropods. The mean initial concentration of glyphosate in sampled ground-dwelling arthropods was found to be 5.97 mg a.s./kg f.w. (measured on DAT 1), when applied at an application rate corresponding to 2.88 kg a.s./ha. The TWA of glyphosate in ground dwelling arthropods for the whole sampling period (DAT 1-35) was found to be 1.64 mg a.s./kg f.w. The residues per unit dose (RUD) for the mean initial residue in ground dwelling arthropods was 2.08. RUD for the maximum 21-day TWA was 0.79.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	MON 52276 (Soluble concentrate)
Active substance:	Glyphosate acid 360 g glyphosate acid equivalents/L (nominal)
Active substance content:	358.8 g glyphosate acid equivalents/L (according to the Certificate of Analysis)
Proposed use:	Herbicide
Description:	Liquid, yellowish to brown
Lot/Batch #:	A9K0106104
Density:	1.1693 g/mL at 20°C (according to the Certificate of Analysis)

2. Vehicle and/or positive control: None

3. Test organism:

Species:	Ground-dwelling arthropods (all field species)
Age:	Not stated

4. Study location:

Study site:	[redacted] Germany
Study field:	Arable field (4.9 ha)
Crop cultivated:	Maize
Soil type:	Coarse loamy sand
Soil composition:	82.0% sand, 11.5% silt, 6.5% clay, 1.9% organic matter
Soil pH:	6

5. Environmental conditions:

Temperature:	-1.6°C - + 23.7°C, (mean = 8.7°C) (non GLP)
Precipitation:	Precipitation was measure daily (non GLP). Most precipitation occurred at the second half of the sampling period. At the day of application and the following three days no precipitation occurred.

B: STUDY DESIGN AND METHODS

1. Experimental treatments:

Study site: The field study was conducted in a maize field situated in [redacted] north Germany, a typical area of maize cultivation in Europe. The study field was typical of maize fields in this region with regard to size (approx. 7.6 ha, but only 4.9 ha were used as sampling area) and basic structure (i.e. line distance). The sampling area was divided into three study plots (approx. 1.6 ha).From the edge of the sampling area at least 14 metres were excluded from sampling procedures to minimise edge effects.

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Test item application: The test item MON was applied on the study plots on 16 April 2010 in accordance with Good Agricultural Practice (nominal application rate of 8 L formulation/ha in a spray volume of 200 L water /ha (corresponding to 2.88 kg a.s./ha).

Arthropod sampling: Samples of the natural population of ground-dwelling arthropods were collected for residue analysis by pitfall trapping. 100 traps per plot were placed at six metre intervals in lines (transects) within the rows of the plant. Each study plot consisted of two transects with 50 traps. To avoid glyphosate contamination of the traps during spray application, the traps were protected by an appropriate cover. The traps were opened approximately 24 h before arthropods were collected. Per sampling day the content of all traps of a plot were pooled. Arthropod samples were collected at DAT (day after treatment) 0 (before application), 1, 2, 3, 5, 7, 9, 11, 14, 17, 21, 28, and 35. Following each sampling event, arthropods were recovered from all pitfall traps on each study plot and the individual samples pooled to provide a single sample for each study plot (replicate).

2. Observations: Directly after collection, the composition of arthropod samples (i.e. all samples from a single study plot) was determined in terms of main taxonomic groups and subdivided into adults and larval stages. All individuals of each group were counted and the fresh weight of each taxonomic group was documented. Then, arthropods from each study plot were re-pooled in polyethylene bottles and stored in the freezer at ≤-18 °C.

3. Residues analysis: Analysis of glyphosate in arthropod samples was conducted by HPLC-MS/MS after extraction and derivatisation as FMOC-glyphosate.

4. Calculations: The initial and maximum concentrations, as well as the 90th percentile of glyphosate concentrations in ground-dwelling arthropods were determined based on single concentrations (n = 3 study plots, with the exception of DAT 5: n = 2 study plots). Furthermore, the mean residue concentrations of glyphosate over a given time period were used to calculate the TWAs for a 21-day period and over the whole study period (DAT 1-35). Residues per unit dose (RUDs) were calculated for the initial residue values and for the maximum 21-day TWA.

II. RESULTS AND DISCUSSION

A. FINDINGS

The residue data of glyphosate in arthropods are presented below.

Table 8.16.2-1 Mean concentrations of glyphosate residues in ground-dwelling arthropods

Mean concentrations of glyphosate residues [mg a.s./kg f.w.] in ground-dwelling arthropods(n=3; except for DAT 5: n=2)		**Residues per unit dose(RUD)**
Initial concentration	5.97 (DAT 1)	2.08 (DAT1)
Maximum concentration over sampling period	5.97 (DAT 1)	-
Maximum 90th percentile	7.30 (DAT 2)	-
TWA (21-day period)	2.26 (DAT 1-21)	0.79 (DAT 1-21)
TWA (entire study period) long-term risk	1.64 (DAT 1-35)	-

DAT day after treatment

On DAT 5 the content of all samples of study plot 2 had to be discarded, because melting water flooded the traps after a shower of sleet.

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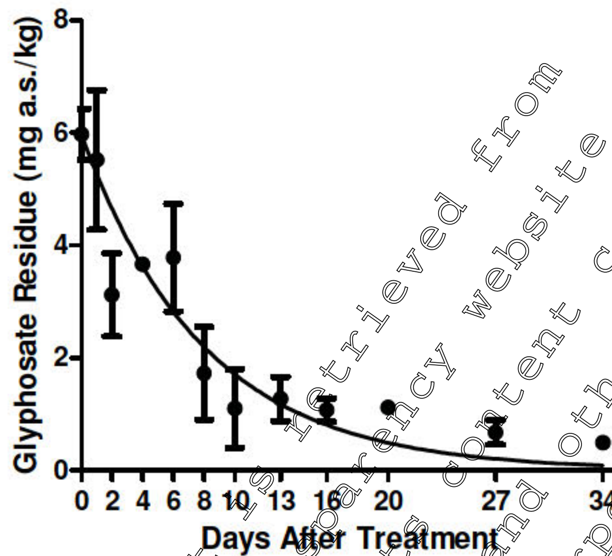
B. OBSERVATIONS

The observed time course for glyphosate residue decline was rapid. The time-dependent decline is illustrated below. Glyphosate has a low log K_{ow}, does not bio accumulate, and the residue decline curve demonstrated first order exponential decline (see below). The DT_{50} for glyphosate residue was estimated to be approximately 5.5 days. This finding is consistent with the rapid decline that has been observed in foliage, where the grass DT_{50} for glyphosate residue decline for was estimated to be approximately 3 days.



Climatic conditions at the test site during the sampling period were favourable for the conduct of this study, no rain occurred at the day of application and the first half of the sampling period was very dry.

III. CONCLUSION

The study provides realistic data on the magnitude of peak residue levels and the corresponding time course of residue decline of glyphosate in ground-dwelling arthropods. Glyphosate residues rapidly declined with a DT_{50} of approximately 5.5 days. Maximum mean concentrations of glyphosate in ground-dwelling arthropods (mean, n = 3, with the exception of DAT 5: n = 2) over the sampling period were 5.97 mg a.s./kg f.w. (measured on DAT 1), when applied at an application rate corresponding to 2.88 kg a.s./ha.

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Ecotoxicology Literature Review

Overview of Searching methodology

Monsanto Company has been conducting routine surveillance of technical literature for glyphosate-related publications in a structured fashion since early 1997. During the period from 1997 to the present time, the search process and the literature databases used have been modified as new resources and technology became readily available. The technical databases that are used for the search include: Web of Science℠, BIOSIS Previews®, CAB Abstracts® (CABI), MEDLINE®, and CA Plus (Chemical Abstracts Plus). The searches are done on glyphosate acid, glyphosate salts (including isopropyl amine, potassium, ammonium, and methylamine), and AMPA, and their related chemical names and CAS numbers. Searches based on these search terms will also identify publications that consider glyphosate and surfactants, (such as polyoxyethylenealkylamines, or POEA), in the context of glyphosate formulations.

Starting from the ongoing Monsanto literature database, all the peer-reviewed publications covering the time period from 2001 through 2011 that relate to the four key disciplines addressing exposure and hazard (toxicology, ecotoxicology, residues and environmental fate) were assessed within the appropriate discipline for inclusion in the literature review for the submission. Some publications address more than one discipline, and are included in each relevant discipline. More recent publications have continued to be reviewed up to shortly before submission, and selected publications have been included.

At the request of the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), additional publications cited in a recent document prepared by Earth Open Source[9] have also been included in the literature review. Many of the cited peer-reviewed publications were already included, but others were not within the scope of this literature review, primarily because the publication date was prior to 2001. The additional peer-reviewed publications have been included and are discussed within the appropriate discipline.

The peer-reviewed publications identified for inclusion during the literature search were reviewed within each discipline and classified into one of the categories listed below.

- **Category 0 publications**: These are publications in which glyphosate is only mentioned as an example substance or is discussed/studied in a context that is not relevant or related to any of the regulatory sections or the exposure/hazard assessments within this submission; the publication is therefore outside of the scope of this submission.
- **Category 1 publications**: These are publications which discuss glyphosate in a context relevant or related to the regulatory dossier sections and the conclusions fall within the conclusions of the exposure/hazard assessment. The publication is submitted with minimal or no comment or discussion.
- **Category 2 publications**: These are publications which discuss glyphosate in a context relevant or related to the regulatory dossier sections and have conclusions that call into question the endpoints/conclusions in the exposure/hazard assessment. Additionally, Category 2 also includes publications with conclusions that support the risk/hazard assessment, and may be included in discussion of other relevant publications. For selected Category 2 publications, an OECD Tier-II type summary is provided in addition to a reliability assessment (Klimisch rating, see Klimisch et al. 1997); limited comments and critical remarks are provided, as appropriate.
- **Category 3 publications**: These are publications that discuss glyphosate in a context relevant or related to (1) non-regulatory endpoints that need to be addressed as per new Regulation (EC) 1107/2009; or (2) in a context relevant to sensitive allegations that have emerged or could emerge

[9] Earth Open Source report. 2011. Roundup and birth defects: Is the public being kept in the dark? Authored by Antoniou M, Habib MEEM, Howard CV, Jennings RC, Leifert C, Nodari RO, C Robinson, Fagan J. Available from: http://www.earthopensource.org/files/pdfs/Roundup-and-birth-defects/RoundupandBirthDefectsv5.pdf

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in the media; or (3) in a context relevant to the regulatory dossier sections and have conclusions that are in disagreement with endpoints/conclusions in the exposure/hazard assessment (although the experimental design seems relevant at first glance). An OECD Tier-II type summary is provided and a Klimisch rating assigned, and supplemented with critical review and discussion.

- **Category ‘E’ publications**: These are peer-reviewed publications that were cited in the Earth Open Source document. This categoryincludes publications that were already captured by the literature search and are addressed within the appropriate discipline, as well as publications that were out of scope of the search (primarily as a result of being published prior to 2001). Publications already captured in the literature search were assigned a Category 1, 2 or 3 rating (as appropriate) in addition to a Category ‘E’ rating. An OECD Tier-II type summary has been prepared and a Klimisch rating assigned for each of the Category E publications. All Category ‘E’ publications are reviewed within the appropriate discipline, with most of the reviews provided within the toxicology dossier under Section IIA 5.10.

Approximately 2000 peer-reviewed publications from the Monsanto technical literature database were assessed, and of those about 1000 were assigned a Category 1, 2 or 3 and selected for inclusion in the submission.

A full description of the literature search methodology is provided in a separate document (Carr and Bleeke, 2012).

The publications selected for inclusion are listed in Document L for each respective section, under the Annex point for ‘Other/Special Studies’: Point IIA 5.10 (Toxicology), Point IIA 6.10 (Metabolism and Residue), Point IIA 7.13 (Environmental Fate), and Point IIA 8.16 (Ecotoxicology). Under each point, the list of Other/Special Studies is presented in three tables:

- Table 1 lists other relevant studies conducted by the Glyphosate Task Force or member companies in support of the submission, that do not fit within any other dossier points .
- Table 2 lists all the relevant peer-reviewed publications from the literature that were selected for inclusion in the submission. For each publication it is noted whether or not a Klimisch rating is included in the review.
- Table 3 lists the publications and other documents that are cited within the discussion of the literature. These include documents such as government or company reports; publications that are included in the literature review under another section of the dossier; and publications that are outside the scope of the literature review.

Organization of the Ecotoxicology Literature Review

As discussed above, since the 2001 EU glyphosate evaluation, a number of studies testing various wildlife taxa with glyphosate and commercial glyphosate-based formulations have been published.

Articles categorized as a ‘3’ from the ecotoxicological literature have been reviewed for reliability and relevance/adequacy for use in a glyphosate ecological risk assessment using the systematic criteria established by Klimisch et al. (1997). The reliability of a given study was evaluated based on proper determination of the test material, the description of the experimental design, whether standard methodology was used, the validity of the methodology, analytical analyses or lack thereof, if appropriate negative/positive controls were included, reporting of environmental test conditions and having these conditions within acceptable limits as well as the appropriateness of the data analysis. Overall, relevance and adequacy for risk assessment was evaluated based on the relevance of the test material, the relevance of the route of exposure, relevance of test duration, relevance of exposure concentrations, relevance of the taxa or taxon tested, and the extent to which data and/or test designs are appropriate for a particular hazard

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identification or risk characterization. Many of the papers reviewed can be considered as proof of concept. They are mechanistic studies that falls into the category of hazard identification. Hazard identification alone tells us nothing about exposure, and therefore nothing about risk. Furthermore, risk management should not be predicated on hazard identification alone.

In addition to the Klimisch evaluations, extended comments have been prepared for selected publications that provide additional analyses, and/or place results into the context of a risk assessment or into a weight of evidence (WoE) evaluation.

The Klimisch evaluations for glyphosate and glyphosate-based formulations have been divided into the following sub-sections.

- Literature on birds
- Literature on fish
- Literature on amphibians
- Literature on aquatic invertebrates
- Literature on aquatic plants including algae
- Literature on non-target arthropods
- Literature on earthworms
- Literature on soil microbes
- Literature on glyphosate effects on plant disease and nutrient status
- Literature on nontarget plants
- Overview of POEA
- Exposure and effects of POEA to aquatic organisms
- Assessment of concentration addition between glyphosate and surfactants
- Assessment of potential for endocrine activity of glyphosate to wildlife
- An evaluation of developmental and reproductive toxicity and endocrine disruption publications

At the beginning of each section is a preface that provides an introduction to the subject matter.

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Literature on Birds

An extensive regulatory bird toxicology database is available to assess acute, short-term and long-term effects of glyphosate, salts of glyphosate, the metabolite AMPA and the lead glyphosate-based formulation MON 52276 to birds. Glyphosate acid and relevant glyphosate salts (as demonstrated by glyphosate IPA and K salts) have low acute oral toxicity to birds and no mortality was observed in the limit dose studies. In total, four one-generation avian reproduction studies, two with bobwhite and two with mallard duck that meet the requirements of the current OECD one-generation reproduction guideline, have been performed. Two of these four studies, one mallard and one bobwhite reproduction, were not evaluated in the EU 2001 glyphosate evaluation. The NOAEC values for these four one-generation reproduction studies, conducted with two species, were the highest dose tested and demonstrate no long-term effects of glyphosate on reproductive and developmental endpoints.

In this section only one study is reviewed that tested a Roundup formulation, not glyphosate, by repeated oral gavage which is not practiced in avian hazard studies.

Author(s)	Year	Study title
Oliveira, A.G., Telles, L.F., Hess, R.A., Mahecha, G.A.B., Oliveira, C.A.	2007	Effects of the herbicide Roundup on the epididymal region of drakes *Anas platyrhynchos* Reproductive Toxicity. Volume: 23 Issue: 2 Pages: 182-191 DOI: 10.1016/j.reprotox.2006.11.004 ISSN: 0890-6238

Abstract[10]

Exposure to the Roundup has been shown to affect StAR protein and aromatase expression and activity, pointing out that this herbicide may cause adverse effects in animal reproduction by affecting androgen and estrogen synthesis. We tested this hypothesis by investigating the *in vivo* effects of the Roundup on the testis and epididymal region of drake *Anas platyrhynchos*. The exposure to the herbicide resulted in alterations in the structure of the testis and epididymal region as well as in the serum levels of testosterone and estradiol, with changes in the expression of androgen receptors restricted to the testis. The harmful effects were more conspicuous in the proximal efferent ductules and epididymal ducts, suggesting higher sensitivity of these segments among the male genital organs. The effects were mostly dose dependent, indicating that this herbicide may cause disorder in the morphophysiology of the male genital system of animals.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® (not specified further)
Active substance(s):	360 g/L glyphosate, 480 g/L isopropylamine salt
Adjuvant / Surfactant:	Not stated
Description:	Not stated
Source of test substance:	Monsanto do Brasil Ltda, Sao Paulo, Brazil

[10] Quoted from article

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Lot/Batch #: Not stated

Purity: Not stated

Stock solution: Roundup was diluted in distilled water, ratio/dose not specified further

2. Vehicle and/or positive control: Vehicle: distilled water

3. Test organism:

Species: *Anas platyrhynchos* (Mallard duck)

Age of test organisms at study initiation: Adult (age not specified)

Source: 'commercial sources' but no specific given

Holding conditions prior to test: Housed at the facilities of Federal University of Minas Gerais under natural conditions of light, humidity and temperature with free access to water and food

Acclimatisation: **None.** There was no discussion about the acclimation period (i.e., how long the animals were held prior to testing). For guideline studies, birds must be housed for at least two weeks prior to testing, and can be used in definitive testing only if fewer than 10% die or are moribund during acclimation.

4. Test system:

Study type: Laboratory histological study

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 15 days

Test conditions: Glyphosate were applied by oral gavage for 15 consecutive days by dissolving test item concentrations in distilled water. After treatment period, animals were anesthetized, weighed and testes and epididymal region were dissected out.

Treatments: 2 glyphosate concentrations and 1 control fed with distilled water.

Test concentrations: 5 and 100 mg/kg bw but it is not stated mg of what. a.s? formulation?

Replicates per treatment: 1

Organisms per replicate: 6

Feeding during experiments: Not stated

Parameters measured: Body weight, weight of testis, volumetric density of epithelium and lumen of seminiferous tubules, morphometry of testis, histochemistry of ductules and ducts of epididymal region (lysosomes, lipids), hormone levels (testosterone and estradiol)

Analytical determination of test concentrations: None

Validity criteria: None

5. Environmental conditions: Not specified

KLIMISCH EVALUATION

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1. Reliability of study: **Not reliable**.

Comment:

- The study is based on a flawed premise. The authors state that the reason by doing this investigation *in vivo* is that "*Roundup has been shown to affect StAR protein and aromatase expression and activity, pointing out that this herbicide may cause adverse effects in animal reproduction by affecting androgen and estrogen synthesis*." An alleged impact on the StAR protein was published by Walsh et al. In 2000. However, Levine *et al.* (2007) in a follow up study to the Walsh study, investigated the potential role of the surfactant in a Roundup-branded formulation in the inhibition of progesterone production upon treatment of MA-10 mouse Leydig cells. In this study, MA-10 cells were exposed for two hours to various surfactants found in personal and home care products (LAS D-40 [a linear alkylbenzene sulfonate], alcohol ethoxylate, lauryl sulfate [SDS], and benzalkonium chloride), as well as a concentrated Roundup-branded Lawn and Garden herbicide (with 180 g/L glyphosate isopropylamine, and 6.53 g/L surfactant [primarily POEA]), and Roundup blank (formulation without glyphosate). Both the Roundup-branded formulation and Roundup blank decreased the hCG-stimulated increase in progesterone production. In both cases, the median inhibition concentration (IC_{50}) was approximately 5 mg/mL. IC_{50} values for the four other surfactants were similar to that of the Roundup branded formulation and Roundup blank, indicating that: 1) the effect on progesterone is largely attributable to the surfactant, and not glyphosate; and 2) surfactants, in general, decrease hCG-stimulated progesterone production. The impact of the various surfactants on StAR protein levels was also assessed by Western Blot analysis on hCG-stimulated and non-stimulated MA-10 cells. Exposure to the surfactants, Roundup-branded formulation, and Roundup blank resulted in decreased levels of the 30 kDa form of StAR protein, but not the 37 kDa precursor form. Because formation of the 30 kDa form requires mitochondrial import and processing of the 37 kDa precursor, the effect of treatment on mitochondrial potential, an indicator of proper mitochondrial membrane function, was measured using the JC-1 cationic dye. Treated MA-10 cells demonstrated a loss of normal mitochondrial membrane potential, meaning that proper import and processing of the 37 kDa form of the StAR protein was disrupted upon treatment. This finding explains the previously observed decrease in the 30 kDa form of the StAR protein. Additionally, this effect on mitochondrial membrane potential was noted for benzalkonium chloride and the alcohol ethoxylate surfactants, the Roundup branded formulation, and Roundup blank at concentrations below those that affect steroidogenesis. Overall, these results strongly support the concept that the adverse effects of Roundup branded herbicidal formulations on steroidogenesis are not mediated by glyphosate exposure, but rather, by the effect of surfactants on unprotected cells

in culture.

- Results are based on administration of test item by oral gavage, not a dietary exposure which is most representative of an environmentally realistic field exposure.
- The birds in this study were administered Roundup via oral gavage for 15 continuous days. This is not a procedure that is used in avian toxicology and for exposure of this duration a dietary exposure is always used for its realism and for annual welfare issues. This is not a route or duration of exposure that birds are likely to experience in the field. The laboratory acute oral LD_{50} study generally is conducted with a single dose via oral gavage, but this endpoint is used as a comparative measure of effect or to mimic gorge feeding, which would not be expected to occur over multiple days of feeding. There can be significant stress to birds that are handled and dosed frequently as was done in the Oliveira et al. study.
- How birds were fed over the 15 day experiment was not provided.
- Only six birds per treatment and only two treatments (besides the control) were used in this study. The low number of animals brings into question the ability of this study to predict any biologically meaningful adverse effect. Since only two treatments were evaluated, no clear dose-response can be obtained from this study. Interestingly, of the original 6 animals per treatment, only 5 animals per treatment were analyzed for body and testicular weights at the end of the study, but there is no mention of why this was the case or what happened to the additional drake in each treatment. Additionally, it appears that only three of the six drakes per treatment were used to determine plasma testosterone and estradiol levels. There is no mention of which three drakes per treatment level were chosen for determination of hormone levels and why not all six drakes per treatment were used. If there was a legitimate reason for not using blood samples from all of the drakes, it should have been explicitly stated in the publication.

2. Relevance of study:

Comment:

- Study is based on administration of test item by <u>oral gavage</u> not a dietary exposure for 15 days, exceeding a realistic exposure scenario by far. This metholodology is notused in avian toxicology and questions the relevance of this study.
- It remains unclear what dose was administered to the birds, therefore it is not possible to reproduce whether findings are within the range of values previously determined using GLP studies.
- Hormone levels were determined immediately after application, i.e., it is not possible to evaluate whether effects are persistent or ephemeral.
- This finding is not in agreement with the existing weight of evidence from 2 standard avian one generation reproduction studies conducted with glyphosate acid.

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- During the phase 3 validation of the OECD steroidogenesis assay, glyphosate was shown not to have any effect on estrogen and testosterone production in H295R cells (Hecker et al. 2011).
- Additionally, glyphosate was shown not to effect estrogenic endpoints in the validated OECD Uterotrophic and androgenic endpoints including 5α-reductase activity in the OECD validated Hershberger assay (Saltmiras et al. 2012).

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

References

Levine SL, Han Z, Liu J, Farmer DR, Papadopoulos V. (2007). Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. Cell Biol Toxicol. 2007 Nov;23(6):385-400.

Hecker M, Hollert H, Cooper R, Vinggaard AM, Akahori Y, Murphy M, Nellemann C, Higley E, Newsted J, Laskey J, Buckalew A, Grund S, Maletz S, Giesy J, Timm G. (2011). The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study. Environ Sci Pollut Res Int. 18(3):503-15.

Saltmiras D, Tobia A. 2012. No evidence of endocrine disruption by glyphosate in Hershberger and Uterotrophic assays (conference abstract). Abstract PS 2198. The Toxicologist (supplement to Toxicological Sciences)126(1): 474. http://www.toxicology.org/AI/PUB/Toxicologist12.pdf

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Literature on Fish

Glyphosate formulations intended for agricultural uses are generally composed of two major components: (1) the active ingredient glyphosate in ionized form with a positively charged counterion such as isopropylamine, dimethylamine, potassium, or ammonium, and (2) a surfactant to facilitate absorption of the polar molecule glyphosate through the waxy cuticle of plant leaf surfaces and into plant cells so that glyphosate can be transported to its site of action in plant chloroplasts. While many surfactants have been tested, there are a relatively limited number of surfactant classes that facilitate glyphosate uptake and consequently provide effective weed control under the environmental conditions present in the European Union. However, products designated for aquatic weed control may contain only glyphosate in its salt form with instructions to select the appropriate surfactant to mix with glyphosate prior to application. In either case, the final liquid to be sprayed contains glyphosate in salt form and a surfactant. While both components are present in the final liquid that is sprayed, the properties of each component, glyphosate and surfactant, will determine the environmental toxicity.

It is well established in the scientific literature that surfactants can increase the permeability of biological membranes. With aquatic vertebrates and invertebrates increased permeability of gill epithelial membranes is the non-specific toxic mode of action and results in hypoxia and loss of osmotic or ionic stability occurring at the gill. For this reason, similar acute toxicity values are obtained for aquatic vertebrates and aquatic invertebrates with gills. However, surfactant effects observed in acute and chronic laboratory experiments, and conducted under continuous exposure, must be evaluated with caution because the environmental fate of the compound has not been included in the exposure regime.

In this section, a series of articles that have primarily evaluated the effect of glyphosate-based formulations has been reviewed for reliability and relevance/adequacy for risk assessment. However, a portion of the studies tested glyphosate alone. For many of the published studies it is unclear what formulation was tested since specific details are not provided. Endpoints evaluated in many of these studies include survival, growth, histopathology and various biochemical endpoints. In many of these papers, evaluations on sub-lethal endpoints were performed on animals exposed to concentrations at or near the acute LC_{50} values; these levels greatly exceed realistic environmental concentrations by one to two orders off magnitude.

Examination of the ratio of LC_{50} to NOEC values for studies evaluating effects of glyphosate acid or glyphosate formulations on fish species indicates that the mean LC_{50} to NOEC ratio approximately 2 for studies in which a NOEC value was reported. A ratio of generally about 2 is indicative of a relatively steep slope (narrow concentration response range) for the concentration-response curve. The TER threshold is 100 for aquatic species. This threshold includes an uncertainty consideration for the limited number of required surrogate species tested and does not account for the significant additional certainty gained when having results from a large number of fish species. Consequently, the TER value for aquatic animal assessments can be less than 100 based on all of the testing performed on glyphosate formulations and the steep slope for concentration-effect curves.

In section IIA 8.2 acute and chronic toxicity to fish has been summarized. Glyphosate has low acute toxicity to fish and typically the pH effect of high concentrations of glyphosate is what results in toxicity. The difference in buffering potential between diluent waters for various test laboratories is apparently one of the key factors contributing to the intra- and inter-species variation in tolerance to glyphosate. Additionally, several chronic studies have been conducted for glyphosate and fish. Among these is a fish full life cycle (FFLC) study performed with the fathead minnow (*Pimphales promelas*), which is standard freshwater test species for this study type. The fish full life-cycle study is the highest Tier of fish ecotoxicology testing. In the study, the entire life-cycle is exposed; therefore, *de facto* the most sensitive life-stage to any toxicant (including endocrine active compounds) is encompassed by the test design. In

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the first or parental generation (F0) the following endpoints are evaluated: embryo hatching success, embryo time-to-hatch, survival at various time points after hatch, length and wet weight as an indicator of growth at various time points after hatch, and reproductive success (fecundity) as measured by number of eggs per female per day, number of spawns and number of eggs per spawn. In the second generation (F1) the endpoints include embryo hatching success, embryo time-to-hatch, survival, and development as measured by length and wet weight at specified time points.

The full life-cycle study with fathead minnows exposed to glyphosate began with two groups of thirty fertilized eggs incubated in each test aquarium at 25°C. Post hatch, fish were randomly divided into two groups of twenty and distributed to growth chambers until sexual maturity was reached. When secondary sexual characteristics were well developed (circa day 134) the number of fish in each tank was reduced initially to 4 males and 4 females, and subsequently to 2 males and 4 females.

When spawning began, eggs were removed from the underside of spawning tiles daily and eggs of each spawn were counted. Fifty eggs from each of the first ten spawning in each tank were then oscillated in their respective test waters by means of the egg cup and rocker arm apparatus until hatching was completed (3-5 days at 25°C). After 30 days exposure, fry groups were terminated and total lengths determined by the photographic method. Total wet weight and percent survival were also determined at this time for each fry group. Total length, wet weight, sex and gonadal conditions were determined for each adult fish at the termination of the experiment.

Continuous exposure to concentrations of glyphosate as high as 25.7 mg/L had no statistically significant effects on any of the parameters studied during 254 days of continuous exposure. Hatchability of eggs was excellent (>94%) in all concentrations of glyphosate and controls. Per-cent survival and total length of fathead minnows after 30 and 60 days exposure to concentrations of glyphosate as high as 25.7 mg/L did not differ significantly from the control. This result is consistent with regulatory studies that demonstrate a lack of reproductive effects by glyphosate.

The estrogenic potency of glyphosate has also been evaluated *in vivo* with rainbow trout by measuring vitellogenin levels post glyphosate exposure. After seven days of exposure to 0.11 mg/L glyphosate there was no effect on vitellogenin levels, indicating that glyphosate is not estrogenic (Xie et al, 2005). Consistent with this result, Petit et al. (1997) demonstrated that glyphosate does not bind to and transactivate a rainbow trout estrogen receptor.

This section reviews a number of articles that evaluated the toxicity of various glyphosate based formulations on fish survival and a range of sublethal endpoints. A common practice in many of these studies was to evaluate impacts on sub-lethal endpoints that were coincident with mortality at environmentally unrealistic exposure levels and durations and did not consider the environmental fate of the materials tested. Therefore, the results from these studies need to be evaluated with caution.

References

Petit F, LeGoff P, Cravedi J-P, Valotaire Y, Pakdel F. (1997). Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J. Molecular Endocrinology. 19: 321-335.

Xie L, Thrippleton K, Irwin MA, Siemering GS, Mekebri A, Crane D, Berry K, Schlenk D. (2005). Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay. Toxicol Sci. 87:91-8.

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Author(s)	Year	Study title
Filizadeh, Y., Rajabi Islami H.	2011	Toxicity determination of three sturgeon species exposed to glyphosate Iranian Journal of Fisheries Sciences Volume: 10 Issue: 3 Pages: 383-392 Publication not available online DOI: not applicable ISSN: not stated

Abstract[11]

Glyphosate, N–(phosphonomethyl) glycine, has been widely used to control agricultural weeds in the north of Iran. However, it is also supposed to have adverse effects on natural sturgeon population. The present study was undertaken to evaluate the acute toxicity of glyphosate to three different sturgeon species (*Huso huso*, *Acipenser stellatus*, and *A. persicus*) under laboratory conditions. Fish were exposed to one of ten glyphosate concentrations (10 to 100 mg l^{-1} with 10 mg l^{-1} intervals), along with a control group. The values of the median lethal concentration (LC_{50}) for each experimental species were estimated using a standard probit regression analyses after each 6, 12, 24, 48, 96, and 168 hours as exposure times. Results showed that increase in glyphosate exposure times up to 168 hours was simultaneous to decrease of the lethal concentration (LC_{50}). 96–h LC_{50} of glyphosate for *H. huso*, *A. stellatus* and *A. persicus* were 26.4, 23.2 and 27.5 mg l^{-1}, respectively. Glyphosate exhibited a slight to moderate toxicity in sturgeon species. However, it may negatively affect the natural population of sturgeons through decreasing of fry mass, smaller size of yolk sac and the initiation of unsafe behaviours.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® - not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
Active substance(s):	Glyphosate acid
Surfactant:	Not stated
Description:	Not stated
Source of test substance:	Monsanto Company, St. Louis, MO
Lot/Batch #:	Not stated
Purity:	41% a.e.
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	*Huso huso, Acipenser stellatus, A. persicus* fry
Age of test organisms at study initiation:	juvenile
Size:	Length: 8.0 ± 0.4 cm, weight: 10.2 ± 1.0 g
Source:	International Sturgeon Research Institute (ISRI), Rasht, Iran
Holding conditions prior to test:	4 weeks in 400 L tanks with constantly aerated water; fish were fed once daily with commercial fish pellet food, faeces and

[11] Quoted from article

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pellet remains were removed daily.

4. Test system:

Study type:	Static
Guideline:	None; study design comparable to OECD 203
GLP:	No
Guideline deviations:	none
Duration of study:	168 h
Test conditions:	Tests were conducted in 100 L aquariums.
Replicates per concentration:	3
Organisms per replicate:	8
Feeding:	None
Parameters measured:	Mortality at 6, 12, 24, 48, 96 and 168 hours, water quality parameters and behavioural changes were monitored daily. Mortality data were evaluated using probit regression analysis. Differences between species were evaluated using one-way-ANOVA followed by Tukey's HSD test.
Test concentrations:	10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg Roundup®/L (nominal) and water control
Analytical determination of test concentrations:	Not measured

5. Environmental conditions:

Test medium:	Not stated
Temperature:	22 ± 1.5 °C
Photoperiod:	12 h light
Light intensity	Not stated
pH:	7.4 ± 0.2
Dissolved oxygen	6.8 ± 1.3 mg/L
Ammonium:	0.009 ± 0.004 mg/L
Nitrite:	0.02 ± 0.01 mg/L
Nitrate:	0.63 mg/L

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment:

- Clear description of the test substance missing; not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
- Experimental details missing (test medium)
- No analytical measure performed

2. Relevance of study: **Not relevant**

Comment: A clear description or specification of the test substance is missing and this is a serious flaw; no content of active substance is stated. It is not clear what units the endpoints are expressed in. The endpoints (acute LC_{50} values) are seemingly

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within the range expected for glyphosate and formulations containing glyphosate, therefore, current manuscript does not provide relevant new findings.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

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Author(s)	Year	Study title
Guilherme, S., Gaivãob, I., Santosa, M. A., Pachecoa, M.	2012	DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide – Elucidation of organ-specificity and the role of oxidative stress. Journal: Mutation Research/Genetic Toxicology and Environmental Mutagenesis Volume: 743 Issue: 1-2 Pages: 1-9 DOI: 10.1016/j.mrgentox.2011.10.017 ISSN: 1383-5718

Abstract[12]

Organophosphate herbicides are among the most dangerous agrochemicals for the aquatic environment. In this context, Roundup®, a glyphosate-based herbicide, has been widely detected in natural water bodies, representing a potential threat to non-target organisms, namely fish. Thus, the main goal of the present study was to evaluate the genotoxic potential of Roundup® in the teleost fish *Anguilla anguilla*, addressing the possible causative involvement of oxidative stress. Fish were exposed to environmentally realistic concentrations of this herbicide (58 and 116 g L−1) during one or three days. The standard procedure of the comet assay was applied to gill and liver cells in order to determine organ-specific genetic damage. Since liver is a central organ in xenobiotic metabolism, nucleoids of hepatic cells were also incubated with a lesion-specific repair enzyme (formamidopyrimidine DNA glycosylase – FPG), in order to recognise oxidised purines. Antioxidants were determined in both organs as indicators of pro-oxidant state. In general, both organs displayed an increase in DNA damage for the two Roundup® concentrations and exposure times, although liver showed to be less susceptible to the lower concentration. The enzymemodified comet assay showed the occurrence of FPG-sensitive sites in liver only after a 3-day exposure to the higher Roundup® concentration. The antioxidant defences were in general unresponsive, despite a single increment of catalase activity in gills (116 g L−1, 3-day) and a decrease of superoxide dismutase activity in liver (58 ug L−1, 3-day). Overall, the mechanisms involved in Roundup®-induced DNA strandbreaks showed to be similar in both organs. Nevertheless, it was demonstrated that the type of DNA damage varies with the concentration and exposure duration. Hence, after 1-day exposure, an increase on pro-oxidant state is not a necessary condition for the induction of DNA-damaging effects of Roundup®. By increasing the duration of exposure to three days, ROS-dependent processes gained preponderance as a mechanism of DNA-damage induction in the higher concentration.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® Ultra
Active substance(s):	Glyphosate (isopropylammonium salt of glyphosate)
Surfactant:	Polyethoxylene amine but was not confirmed by the authors
Description:	None
Source of test substance:	Distributed by Bayer CropScience, Portugal
Lot/Batch #:	Not stated
Purity:	485 g L^{-1} as the active ingredient (equivalent to 360 g L^{-1} or

[12] Quoted from article

30.8% of glyphosate) and polyethoxylene amine (16%)

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Anguilla anguilla* L. (Eel)

Age/stage/weight of test organisms at study initiation: Average length of 25 ± 3 cm and weight of 32 ± 5 g (yellow eel stage)

Source: Captured from an unpolluted area of the Aveiro Lagoon – Murtosa, Portugal

Holding conditions prior to test: The eels kept in 80-L aquaria under a natural photoperiod, in aerated, filtered, de-chlorinated and recirculating tap water, with the following physico-chemical conditions: salinity 0, temperature 20 ± 1 °C, pH 7.3 ± 0.2, ammonia <0.1 mg L^{-1}, nitrite 0.06 ± 0.03 mg L^{-1}, nitrate 25 ± 6.0 mg L^{-1}, dissolved oxygen 8.1 ± 0.5 mg L^{-1}.
During this period, fish were fed every other day with fish roe. Fish were not fed one day before the experiment was started.

Acclimatisation: 12 d

4. Test system:

Study type: laboratory, static

Guideline: Not stated

GLP: Not stated

Guideline deviations: -

Duration of study: 3 d

Test conditions: 20 L aquaria, physicochemical characteristics of the water please refer to "Holding conditions prior to test:"

Treatments: 2 (+ control)

Replicates per concentration: 2

Organisms per replicate: 6

Feeding: None

Parameters measured:

- Protein content as indicator for following enzyme activity:
  - Catalase
  - Superoxide dismutase activity (with Ransod kit (Randox Laboratories Ltd., UK)
  - Glutathione-S-transferase
  - Glutathione peroxidase
  - Glutathione reductase
- Genetic damage measured by comet assay

Test concentrations: 58 µg L^{-1} Roundup ® (=18 µg a. i. L^{-1}),
116 µg L^{-1} Roundup ® (=36 µg a. i. L^{-1}),

Application: Not stated

Application devices: Not stated

Application verification: Analytical verification not confirmed

Analytical determination of test concentrations: Not stated

5. Environmental conditions:

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Test medium:	Please refer to "Holding conditions prior to test:"
Temperature:	Please refer to "Holding conditions prior to test:"
Photoperiod:	Natural photoperiod, season or date is unknown
Light intensity:	Not stated
pH:	Please refer to "Holding conditions prior to test:"
Oxygen saturation	Please refer to "Holding conditions prior to test:"
Conductivity:	Please refer to "Holding conditions prior to test:"
Hardness:	Not stated
Alkalinity:	Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:
- The amount of replicates (n =2) per test concentration is insufficient. More test concentrations are essential to derive a dose depending effect on genetic damage.
- Analytical verification not confirmed.

2. Relevance of study: **Not relevant**

Comment**:** Paper starts with the faulty premise that glyphosate is highly toxic because it is an organophosphate insecticide. Glyphosate is not an organophosphate insecticide nor a neurotoxicant. Glyphosate is a glycine herbicide with an amino-acid like structure containing an organophosphonate moiety.

Weight of evidence for in regulatory studies demonstrates glyphosate is not gene toxin.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment**

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Author(s)	Year	Study title
Hued, A.C., Oberhofer, S., de los Angeles Bistoni, M.	2012	Exposure to a Commercial Glyphosate Formulation (Roundup®) Alters Normal Gill and Liver Histology and Affects Male Sexual Activity of *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes) Archives of environmental contamination and toxicology Volume: 62 Number: 1 Pages: 107-117 Url: http://www.springerlink.com/content/0007046332720860/ DOI: 10.1007/s00244-011-9686-7 ISSN: 0090-4341 (Print) 1432-0703 (Online)

Abstract

Roundup is the most popular commercial glyphosate formulation applied in the cultivation of genetically modified glyphosate-resistant crops. The aim of this study was to evaluate the histological lesions of the neotropical native fish, *Jenynsia multidentata*, in response to acute and subchronic exposure to Roundup and to determine if subchronic exposure to the herbicide causes changes in male sexual activity of individuals exposed to a sublethal concentration (0.5 mg/l) for 7 and 28 days. The estimated 96-h LC_{50} was 19.02 mg/l for both male and female fish. Gill and liver histological lesions were evaluated through histopathological indices allowing quantification of the histological damages in fish exposed to different concentrations of the herbicide. Roundup induced different histological alterations in a concentration-dependent manner. In subchronic-exposure tests, Roundup also altered normal histology of the studied organs and caused a significant decrease in the number of copulations and mating success in male fish exposed to the herbicide. It is expected that in natural environments contaminated with Roundup, both general health condition and reproductive success of *J. multidenatata* could be seriously affected.

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MATERIALS AND METHODS

1. Test material:

Test item: Roundup Max granular
Active substance(s): Glyphosate
Source: Monsanto, Argentina
Purity: 74.70 % of glyphosate (reported to contain 25.3% surfactant)
Lot/Batch #: Not reported

2. Vehicle: Water

3. Test animals:

Species: *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes)
Source: Captured with backpack electrofisher equipment from a site on Yuspe River (64°32'W; 31°17'S) (Cordoba, Argentina)
Age of test animals at study initiation: Not reported
Sex: Male and female
No. of fish: Not reported
Body weight: 0.58 ± 0.21 g for males;
1.12 ± 0.5 g for females

Body length: 36.34 ± 4.16 for males;
43.71 ± 7.46 for females

Acclimation period: 15 days

Diet/Food: Fed twice a day with commercial fish food during acclimation.

Housing: Maintained in a 120 L aerated glass aquarium containing dechlorinated tap water

Environmental conditions: Temperature: 21 ± 1°C
12-hour light/dark cycle

4. Test system:

Study type: Short-term and sub-chronic toxicity of a commercial Glyphosate Formulation (Roundup®) in *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes)

Guideline: Non

GLP: No

Guideline deviations: Not applicable

Test conditions:

<u>Short-Term Toxicity Testing</u>

Duration of study: 96 h

Dose levels: Nominal concentrations of Roundup: 5, 10, 20, 35, 60, and 100 mg/l

Animals per dose group: 8 per group (4 ♂, 4 ♀)

Diet/Food: Individuals were starved 24 h prior to the experiment and were not fed during the experiment.

Exposure: The control and test substance groups were exposed to glyphosate via water in 18 L aerated glass aquaria each.
Each control group and test substance group was performed in duplicate.

<u>Subchronic Toxicity Testing</u>

Duration of study: 7 and 28 days

Dose levels: 0.5 mg/l of Roundup (10% of the lowest concentration used in the acute toxicity test)

Animals per dose group: Two groups of nine individuals (five male and four female fish)

Diet/Food: Fish were fed twice a day with commercial fish food.

Exposure: The control and test substance groups were exposed to glyphosate via water in 18 L aerated glass aquaria each. Each control group and test substance group was performed in duplicate.
The water in each aquarium was renewed partially every 2 days, and completely renewed once a week

<u>Male Sexual Activity</u>

Test conditions: After the sub-chronic exposure period, each male fish was introduced into a 10-L aquarium with an unexposed sexually mature female fish.

5. Observations/analyses:

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Test substance preparations: Stability, achieved concentrations, homogeneity not reported

Mortality: Fish without respiratory movements and no response to tactile stimulus were considered dead and removed immediately.

Sacrifice/pathology: After 96 h of exposure, the surviving fish, as well as those male and female fish exposed to the sublethal concentration for 7 and 28 days, were killed with an overdose of tricaine methane sulfonate and dissected.

Histology and morphometry: Histological Analysis
The liver and gills of surviving Control and exposed fish after 96 h or sub-chronic exposure for 7 and 28 days were examined for tissue lesions. For histopathological analysis of gills, 5 filaments per individual fish were examined.

Measurements: Short-Term Toxicity Testing
The median lethal concentrations (LC50) at 96 hours were estimated separately for male and female fish by a probit transformation from the mortality dose curve using free Probit software (version 1.5) provided by the United States Environmental Protection Agency.

Male Sexual Activity
Male sexual activity was registered using a digital camera (model no. DSC-W70; Sony) and by direct observation for 20 min with observations starting 10 min after the couple was introduced into the tank.
Based on the normal reproductive behavior, the following parameters were estimated:
1. Number of persecutions (NP): number of times that a male fish persecutes a female fish to make contact with the female gonopore.
2. Copulation attempts (CA): number of times that a male fish enlarges its gonopodium to make contact with the female gonopore.
3. Number of copulations (C): number of times that a male fish made direct contact through its gonopodium with the female gonopore.
4. Mating success (MS): estimated from the abovementioned parameters through the following formula: MS = log C/(log NP + log CA); this estimate gives an idea of the effectiveness of a male fish to copulate after persecution or copulation attempts.

Statistics: Data distributions were analyzed using the Shapiro–Wilks index (Sokal and Rohlf 1999). The 96-h LC50 values between sexes were assessed with Student t test. To compare the biological parameters among different Roundup concentrations and time of exposure, Kruskal–Wallis test (Sokal and Rolhf 1999) was performed and followed by a Dunn's multiple comparison test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using Infostat Software Package (Infostat 2002).

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Experimental design did not follow a test guideline and the methodology was not validated or ring tested. The

species of fish that was tested has a very peculiar breeding behaviour and is not an ideal candidate to evaluate male courtship behaviours.

- No water quality parameters were reported such as dissolved oxygen, pH, ammonia, hardness, and alkalinity.
- No analytical confirmations were performed.
- The assay did not have any standards for control performance.
- The results from the assessment of male sexual activity have questionable reliability since the assay was not validated, a positive control was not included, and dose effect relationship, including a NOEC was not established. The effects observed in this study may have been the result of frank toxicity experienced from a prolonged concentration to a high concentration of the formulation.

2. Relevance of study: **Not relevant**

Effects on gill histopathology observed after short-term exposures were at concentrations greater than or equal to 5 mg/L formulation. These types of gill effects are not surprising at high formulation concentrations and considering that the gill is the primary site of toxic action for aquatic animals. These exposure concentrations greatly exceed environmentally relevant concentrations. To put this into perspective, The lowest concentration of 5 mg/L exceeds a direct over-spray of a very shallow water body with the maximum terrestrial application rate of glyphosate. Some of the treatments groups were at 50% of the 96 hour LC_{50} or equivalent top the 96 hour LC_{50} value (20 mg/L formulation). The most severe effects were observed at 35 mg/L formulation which likely represents an LC_{75}. In other words, gill histopathology was done on the 25% surviving fish from this treatment.

Effects on liver histopathology observed after short-term exposures were at concentrations greater than or equal to 5 mg/L formulation. These exposure concentrations greatly exceed environmentally relevant concentrations. To put this into perspective, The lowest concentration of 5 mg/L exceeds a direct over-spray of a very shallow water body with the maximum terrestrial application rate of glyphosate. Some of the treatments groups were at 50% of the 96 hour LC_{50} or equivalent top the 96 hour LC_{50} value (20 mg/L formulation). The most severe effects were observed at 35 mg/L formulation which likely represents an LC_{75}. In other words, liver histopathology was done on the 25% surviving fish from this treatment.

Effects on liver histopathology observed after long-term exposures were at concentrations greater than 0.5 mg/L formulation, which has a duration that exceeds relevant chronic exposures.

3. Klimisch code: **3 and not adequate for risk assessment**

Author(s)	Year	Study title
Kelly, D.W., Poulin, R., Tompkins, D.M., Townsend, C.R.	2010	Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival Journal of Applied Ecology Volume: 47 Issue: 2 Pages: 498-504 Url: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2664.2010.01791.x/pdf DOI: 10.1111/j.1365-2664.2010.01791.x ISSN: 1365-2664 (online)

Abstract[13]

1. Anthropogenic pollution and disease can cause both lethal and sub-lethal effects in aquatic species but our understanding of how these stressors interact is often not known. Contaminants can reduce host resistance to disease, but whether hosts are impacted at environmentally relevant concentrations is poorly understood.

2. We investigated the independent and combined effects of exposure to the common herbicide glyphosate and the trematode parasite *Telogaster opisthorchis* on survival and the development of spinal malformations in juvenile *Galaxias anomalus*, a New Zealand freshwater fish. We then investigated how exposure to a glyphosate concentration gradient (0·36, 3·6, 36 mg active ingredient (a.i.) L^{-1}) affected the production and release of the infective cercarial stage of the parasite by its snail intermediate host *Potamopyrgus antipodarum*.

3. Survival of juvenile fish was unaffected by exposure to glyphosate alone (at an environmentally relevant concentration; 0·36 mg a.i. L^{-1}) or by *T. opisthorchis* infection alone. However, simultaneous exposure to infection and glyphosate significantly reduced fish survival.

4. Juvenile fish developed spinal malformations when exposed either to infections alone or to infections and glyphosate, with a trend towards greater severity of spinal malformation after exposure to both stressors.

5. All snails exposed to the highest glyphosate concentration (36 mg a.i. L^{-1}) died within 24 h. Snails exposed to a moderate concentration (3·6 mg a.i. L^{-1}) produced significantly more *T. opisthorchis* cercariae than snails in the control group or the low concentration group (0·36 mg a.i. L^{-1}; the same concentration as in the fish experiment).

6. *Synthesis and applications.* This is the first study to show that parasites and glyphosate can act synergistically on aquatic vertebrates at environmentally relevant concentrations, and that glyphosate might increase the risk of disease in fish. Our results have important implications when identifying risks to aquatic communities and suggest that threshold levels of glyphosate currently set by regulatory authorities do not adequately protect freshwater systems.

MATERIALS AND METHODS

1. Test material:

[13] Quoted from article

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Test item(s):	Glyphosate formulation, not specified
Active substance(s):	Not specified
Surfactant:	10-20% POEA, according to authors
Description:	none
Source of test substance:	Ravensdown, New Zealand
Lot/Batch #:	Not stated
Purity:	Not stated
Stock solution:	Not stated

2. Vehicle and/or positive control: none

3. Test organism:

Species:	*Galaxias anomalus* (fish, host) *Potamopyrgus antipodarum* (snail, intermediate host) *Telogaster opisthorchis* (infective trematode)
Age of test organisms at study initiation:	Fish: 7-8 weeks
Size:	27.2 mm ± 0.2
Source:	Fish: juvenile specimens were collected from site with low infection prevalence (3.3%) in of the Upper Taieri catchment. Snails: collected from small tributary of the Upper Taieri River, South Island, New Zealand in December 2008
Holding conditions prior to test:	Fish: maintained for 5 weeks in large holding tanks and fed with fine commercial pellet food. Snails: housed in aquaria at 12 °C. Infected snails were identified by placing 12 individuals each in 12 well trays, incubation at 16 °C for 24 h and screening for cercariae.
Acclimatisation:	2 days

4. Test system:

Study type:	Not specified
Guideline:	Not stated
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	Fish exposure: 26 days Snail test: 14 days
Test conditions:	1.) Fish were randomly allocated to 2 L aquaria containing two uninfected snails plus standard filtered water (control), a *T. opisthorchis* infection alone (two infected snails), glyphosate formulation and two uninfected snails and combined *T. opisthorchis* and glyphosate treatment (two infected snails). Snails were allowed to shed cercariae naturally. 2.) Snails were randomly allocated to 2 L aquaria containing plus standard filtered water (control), or three different concentrations of glyphosate. Each aquarium was supplied with a 5 cm stem of *Rorrippa nasturtium aquaticum*. after 14 days, specimens were transferred individually to 12 well plates and exposed to intense lighting (intensity not specified) and 14 °C for 24 h.
Replicates per concentration:	Fish test: 8 Snail test: 12

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Organisms per replicate:	4 (fish)
Feeding:	1.)Fish: fine pellets Snails: algal pellets every 3 days 2.) *Rorrippa nasturtium aquaticum* as periphyton substrate for grazing, fresh macrophyte was provided at start of 2nd week
Parameters measured:	1.) Each replicate was monitored twice daily, at the end of the exposure, all fish were assessed for spinal malformation and numbers of metacercariae were counted. Data were compared using ANOVA 2.) Cercariae were counted in six shedding cycles: following one cycle of 24 h of light and temperature stimulation cercariae were counted and snails were allowed to rest for a period of 24 h. At the end of the exposure, snail length was measured. Physico-chemical parameters: not stated.
Test concentrations:	1.) fish test: 0.36 mg a.s./L 2.) snail test: 0.36, 3.6 and 36 mg a.s./L for 10 days
Analytical determination of test concentrations:	Not stated
Validity criteria:	Not applicable

5. Environmental conditions:

Test medium:	Standard filtered water
Temperature:	1.) 12 – 14 °C 2.) 8 °C
Photoperiod:	8 h
Light intensity	Not stated
pH:	Not stated
Oxygen saturation	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (major experimental omissions)

- From the materials and methods description it is unclear, whether POEA was mixed additionally by the authors of the study into the treatments or was already contained in the formulation.
- No data about maintenance of test concentration change of test medium (static, static renewal, flow through conditions) provided. Especially when considering that fish and snails were fed regularly, it is essential to clean the testing vessels several times within a period of 26 days.
- Description of statistics applied is insufficient (post hoc tests not specified).
- As a herbicide, glyphosate may potentially affect *Rorrippa nasturtium aquaticum* contained as food supply in the snail exposure tanks, but no effects are reported.

2. Relevance of study: **Not relevant**

Testing performed with an unknown formulation.

3. Klimisch code: **3 and not adequate for risk assessment**

Expanded comments

During the experiments, fish were exposed to the trematode parasite, the glyphosate formulation, or the glyphosate formulation and the parasitic trematode. Based on the results, the researchers claim a significant effect on fish survival where only the combination of glyphosate formulation and infected snails caused significantly reduced survival compared to controls. When comparing spinal deformations and infection rates in fish, treatments containing infected snails demonstrated increased spinal deformations and increased infection rates in the fish compared to controls; however, there was no significant difference between infected snails alone and infected snails in combination with the glyphosate formulation.

The study of interacting stressors is an important area of research; however, this study is not relevant for the assessment of effects of glyphosate formulations in the environment fundamentally because: the authors inaccurately ascribe the effects they observe to glyphosate, when they did not test glyphosate alone; exposures were not environmentally relevant and no information is provided in the paper on water quality in the exposure tanks.

Additional background

The experiments were conducted to examine the potential effects to a New Zealand fish species, *Galaxias anomalus*, of two stressors – exposure to the trematode parasite, *Telogaster opisthorchis*, and exposure to a glyphosate formulation. Fish were exposed to the trematode parasite, the glyphosate formulation, or the glyphosate formulation + parasitic trematode. The trematode is carried by a snail host (*Potomopyrgus antipodarum*). Exposures were conducted in 2-L aquaria for 26 days. Each treatment was conducted with 8 replicates, each containing 4 fish and 2 snails. The treatments consisted of (1) control (uninfected snails); (2) infected snails; (3) glyphosate formulation with uninfected snails; and (4) glyphosate formulation with infected snails. Nominal glyphosate concentrations were reported as 0.36 mg a.i./L (presumed to be 0.36 mg glyphosate IPA salt/L).

In a second experiment, snails infected with the trematode were exposed to the same glyphosate formulation at concentrations of 0.36, 3.6, and 36 mg a.i./L for 10 days. All snails at the highest concentration died. Release of parasite larvae (cercaria) from the snails was measured for the other two test concentrations. The number of cercaria released from the 0.36 mg a.i./L test concentration was comparable to the number released by the control group exposed to water only. The number of cercaria released from the 3.6 mg a.i./L test concentration was greater than from the control.

The authors inaccurately ascribe the effects they observe to glyphosate, when they did not test glyphosate alone.

- The formulation tested contained both glyphosate and a surfactant (POEA). The effects observed may be due to general surfactant effects, but surfactant alone was not tested.
- Surfactants are present in shampoos, other personal care products, home cleaning products, as well as in many industry applications. None of these products were tested.

Despite the author's claims, exposures were not environmentally relevant.

- Fish and snail exposures to the formulation were much longer (26 and 10 days, respectively) than would occur in the environment.

Glyphosate and POEA bind tightly to sediment and rapidly dissipate from the water phase of natural water/sediment systems.

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- POEA concentrations are reduced to one-half in less than 1 day (Wang *et al.* 2005)
- Glyphosate concentrations are reduced to one-half in 1-4 days (European Commission 2002)

Concentrations cited as environmentally relevant are not from agricultural applications of glyphosate formulations.

- The concentration of 3.7 mg a.i./L is the maximum possible concentration following direct application of 12 – 14 litres/ha of a glyphosate formulation directly to 15 cm of water.
- Annualized mean surface water concentrations predicted from agricultural applications are 0.015 mg a.i./L (0.0114 mg a.e./L, as reported in Giesy, 2000).
- Glyphosate concentrations in samples taken from surface water bodies in agricultural areas did not exceed 0.10 mg a.e./L (0.14 mg a.i./L) and only 3 of 1242 samples exceeded 0.010 mg a.e./L (0.014 mg a.i./L) (Scribner *et al.* 2007)
- In very extreme cases, such as water from overland flow, concentrations in the range of 0.36 mg a.i./L may be present, but this exposure concentration was found to have no effect on parasite larvae release, infection rate in fish, or spinal deviations per fish.

Fish survival was reported to be affected at 0.36 mg a.i. glyphosate formulation/L in the presence of the parasites, but no information is provided in the paper on water quality.

- Basic water quality parameters such as dissolved oxygen, pH, and ammonia concentrations are not reported.
- Fish or snails could have died from low oxygen levels or other toxic by-products present in the tanks from the long exposures.

References:

European Commission, 2002. Review report for the active substance glyphosate. Directive 6511/VI/99, January 21. ttp://ec.europa.eu/food/fs/sfp/ph_ps/pro/eva/existing/list1_glyphosate_en.pdf .

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Kelly DW, Poulin R, Tompkins DM, Townsend CR, 2010. Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. Journal of Applied Ecology. doi: 10.1111/j.1365-2664.2010.01791.x

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Author(s)	Year	Study title
Salbego, J. Pretto, A. Gioda, C.R. de Menezes, C.C. Lazzari, R. Radunz Neto, J. Baldisserotto, B. Loro, V.L.	2010	Herbicide Formulation with Glyphosate Affects Growth, Acetylcholinesterase Activity, and Metabolic and Hematological Parameters in Piava (*Leporinus obtusidens*) Archives of environmental contamination and toxicology Volume: 58 Number: 3 Pages: 740-745

Abstract

The teleost fish *Leporinus obtusidens* (piava) was exposed to different concentrations of Roundup, a commercial herbicide formulation containing glyphosate (0, 1, or 5 mg L^{-1}), for 90 days. Acetylcholinesterase (AChE) activity was verified in brain and muscle. Hepatic and muscular metabolic parameters as well as some haematological parameters were determined. The results showed that brain AChE activity was significantly decreased in fish exposed to 5 mg L^{-1} Roundup, whereas muscular AChE activity was not altered. Both Roundup concentrations significantly decreased liver glycogen without altering the muscle glycogen content. Hepatic glucose levels were reduced only in fish exposed to 5 mg L^{-1} Roundup. Lactate levels in the liver and muscle significantly increased in fish exposed to both Roundup concentrations. Hepatic protein content remained constant at 1 mg L^{-1} but increased at 5 mg L^{-1} Roundup. In the muscle however, protein content decreased with increasing exposure concentration. The herbicide exposure produced a decrease in haematological parameters at both concentrations tested. The majority of observed effects occur at environmental relevant concentrations, and in summary, the results show that Roundup affects brain AChE activity as well as metabolic and hematologic parameters of piavas. Thus, we can suggest that long-term exposure to Roundup causes metabolic disruption in *Leporinus obtusidens*.

MATERIALS AND METHODS

1. Test material:

Test item: Roundup
Active substance(s): Glyphosate (isopropylamine salt)
Source: Monsanto Co., St. Louis, MO, USA
Purity: 48% acid equivalent of the isopropylamine salt of glyphosate
Lot/Batch #: Not reported

2. Vehicle: Water

3. Test animals:

Species: *Leporinus obtusidens*
Source: Fish Culture Laboratory at the Universidade Federal de Santa Maria (UFSM)
Age of test animals at study initiation: Juvenile, not further specified
Sex: Not specified
No. of fish: 180
Body weight: 8.0 ± 0.5g
Body size: 5.0 ± 1.0 cm

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Acclimation period:	10 days	
Diet/Food:	Fed to satiety once a day with commercial fish feed (38% crude protein; Supra, Carazinho, Brazil)	
Water:	Not relevant	
Housing:	250 L continuously aerated tanks with tap water	
Environmental conditions:	Temperature:	22 ± 0.5°C
	pH:	7.4 ± 0.05
	Dissolved oxygen levels:	7.2 ± 0.2 mg L^{-1}
	Nitrite:	0.06 ± 0.01 mg L^{-1}
	Hardness:	32.0 ± 1.15 mg L^{-1} $CaCO_3$
	Alkalinity:	39.0 ± 3.2 mg L^{-1} $CaCO_3$
	Total ammonia:	0.05 ± 0.001 mg L^{-1}
	14-hour light/10-hour dark cycle	

4. Test system:

Study type:	Evaluation of effects on growth, acetylcholinesterase activity, and metabolic and haematological parameters
Guideline:	Non-guideline study
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	90 days
Dose levels:	Control (tap water) 1 mg L^{-1} Roundup 5 mg L^{-1} Roundup
Animals per dose group:	3 treatment groups, 60 animals per group
Test substance preparation/administration:	Roundup (48% acid equivalent of the isopropylamine salt of glyphosate) was administered with the housing water. Based on the estimated half-life of glyphosate, 50% of the water was renewed, and the herbicide was reapplied in the tanks at 4-day intervals to maintain the expected concentration.

5. Observations/analyses:

Concentrations in tanks:	Glyphosate and AMPA-concentrations were monitored during 8 days.
Mortality:	No mortality observed throughout the study
Clinical signs:	Not reported
Body weight:	15 juveniles were collected at random at 30 and 60 days after the beginning of the experiment of weight and length and then returned to the tanks. After 90 days, all remaining juveniles were collected and measured.
Body weight gain:	Measured and reported
Body length:	Measured and reported
Condition factor:	Measured and reported
Food consumptions:	Measured and reported
Haematology:	Measured parameters: ahematocrit, haemoglobin, and total erythrocyte and leucocyte counts.
Clinical chemistry:	Measured parameters: plasma protein
Sacrifice/pathology:	Not reported

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Organ weights: Not reported

Measurements: Tissue protein, liver and muscle glycogen, lactate and soluble sugar in muscle and liver. Acetylcholine esterase (AChE) activity in brain and muscle

Statistics: The homogeneity of variances among groups was tested with the Levene test. One-way analysis of variance (ANOVA) and Tukey multiple-range tests were used. Data were expressed as mean ± standard deviation, and mean differences were considered significant at $p<0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Although analytical measures were performed measure AMPA values were nearly identical to glyphosate levels, which is not possible based on mass balance and what we know about glyphosate degradation. This greatly calls into question the reliability of the exposure information.

Exposure concentration of 1 mg/L and 5 mg/L greatly exceed chronic exposure levels. However, it is not clear if exposure levels are based on formulation units or glyphosate units in the paper. Concentrations are generally reported as glyphosate levels in the paper, which would translate to nominal formulation concentrations of approximately 3.3 and 16.6 mg/L formulation.

No positive control for any of the biomarkers was included to verify the validity of the measurement endpoints.

2. Relevance of study: **Not relevant**

High concentrations tested over a 90-day period which does not represent a realistic exposure level.

Contrary to what is stated in this paper, glyphosate is not a neurotoxicant based on weight of evidence from standard regulatory studies. Neurotoxicity evaluation in section IIA part 5.

3. Klimisch code: **3 and not adequate for risk assessment**

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Author(s)	Year	Study title
Benck Soso, A., Gil Barcellos, L.J., Ranzani-Paiva, M.J., Kreutz, L.C., Mezzalira Quevedo, R., Anziliero, D., Lima, M., Bolognesi da Silva, L., Ritter, F., Calliari Bedin, A., Finco, J.A.	2007	Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundiá (*Rhamdia quelen*) Environmental Toxicology and Pharmacology Volume: 23 Issue: 3 Pages: 308-313 DOI: 10.1016/j.etap.2006.11.008 ISSN: 1382-6689

Abstract[14]

This work was carried out to verify the effect of a glyphosate-based herbicide on Jundiá hormones (cortisol, 17β-estradiol and testosterone), oocyte and swim-up fry production. Earthen ponds containing Jundiá females were contaminated with glyphosate (3.6 mg/L); blood samples were collected from eight females from each treatment immediately before, or at 1, 10, 20, 30 and 40 days following contamination. A typical post-stress rise in cortisol levels was observed at the 20th and 40th days following exposure to glyphosate. At the 40th day, 17β-estradiol was decreased in the exposed females. A similar number of oocytes were stripped out from females from both groups; however, a lower number of viable swim-up fry were obtained from the herbicide exposed females, which also had a higher liver-somatic index (LSI). The results indicate that the presence of glyphosate in water was deleterious to *Rhamdia quelen* reproduction, altering steroid profiles and egg viability.

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® WG
Active substance(s):	640 g/kg
Adjuvant / Surfactant:	Not stated
Description:	Not stated
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	Not stated
Stock solution:	Roundup was diluted in distilled water to final concentration of 3.6 mg/L

2. Vehicle and/or positive control: none

3. Test organism:

Species:	*Rhamdia quelen* (South American catfish)
Age of test organisms at study initiation:	Adult female (age not specified, 2nd reproductive cycle)
Weight:	400 – 600 g
Source:	Not stated
Holding conditions prior to test:	Earthen ponds, 280 m², 1.2 m max. depth, 687 m above sea level. Hatchery-like handling holding conditions (noise, crowding, chasing, daily capturing)
Loading:	0.5 fish/m²
Acclimatisation:	Not applicable

4. Test system:

Study type:	Static mesocosm study with fish
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	40 days.
Test conditions:	Identical to holding conditions. 8 specimens per treatment were sampled prior to first application and 1, 10, 20, 30 and 40 days

[14] Quoted from article

	after initiation of exposure. Fish were anaesthetized with buffered MS222 and blood samples (2-5 mL) were drawn from the caudal vein. Following blood sampling, fish were sacrificed.
Test concentrations:	Applications of 3.6 mg/L each at an interval of 9 days and a control
Replicates per treatment:	8 females were taken for hormone measurements at every sampling (5 successive occasions). For final fertility measurement the number of replicates/females is not stated (“all remaining females”).
Individual per replicate:	s. above
Feeding during experiments:	Daily *ad libitum* with commercial fish pellets (30% crude protein, Alisul Racoes Ltda. Brasil)
Parameters measured:	Cortisol, 17ß-estradiol, testosterone, AST (aspartate aminotransferase) and ALT (alanine aminotransferase) were measured with commercial kits. GSI (gonado-somatic index) and liver somatic index were determined. After 40 days of exposure, fertility was measured: y spawning was induced, oocytes were counted and weighed, and number of swim-up fry produced was determined.
Analytical determination of test concentrations:	None
Validity criteria:	None

5. Environmental conditions:

Test medium:	Not stated
Flow rate:	6 L/min
Temperature:	Not stated
Photoperiod:	Not stated
Light intensity	Not stated
pH:	7.0 – 7.2
Oxygen saturation	5.0 – 7.0 mg/L
Conductivity:	Not stated
Hardness:	45 ± 5 mg $CaCO_3$/L
Alkalinity:	45 ± 5 mg $CaCO_3$/L

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**.

Comment:

- Most critical is the fact that it is not clear to what concentration the fish were exposed to: Although it is claimed that a single concentration of 3.6 mg/L was applied, application was repeated with an interval of 9 days to ‘maintain the expected concentration’. No analytical validation of the exposure concentrations was conducted, so no evidence with regard to ‘real’ exposure concentration is given.
- Possible effects of adjuvants and surfactants of the

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formulation are neglected.

2. Relevance of study: **Not relevant**

Comment:
- Lead formulation MON 52276 was not the test substance and an unrelated formulation in composition was tested. See expanded comments below.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment**

Expanded comments

This study was performed evaluate the effect of a glyphosate-based herbicide on female Jundia (Rhamdia quelen) hormones (cortisol, 17B-estradiol and testosterone), oocyte and swim-up fry production. Exposures were conducted at 50% of the reported 96-hr LC50 (3.6 mg/L) for this species. It is not clear from the paper whether exposure levels are based on units of glyphosate acid equivalents per liter or in mg formulation per liter, but it appears to be the former. The formulation was re-applied on 9 day intervals and biological measurements were made on 10 day intervals (i.e., one day after reapplication). However, glyphosate and/or POEA levels were not measured over the course of the study. This exposure concentration used in this study is an environmentally unrealistic exposure level for the surfactant and exceeds the levels predicted by the exposit model in a water body by orders of magnitude.

An important experimental design flaw in the study is the lack of replication of test and control treatments. Therefore, there was no independence of samples and a statistical analysis of the data was inappropriate (i.e., pseudoreplication). Additionally, temperatures were not reported for the test and control treatments and small differences in temperature between treatments can have a significant impact on the measured endpoints. Further, only the range of certain water quality measurements was reported and it not reported when these measurements were taken.

As stated by the authors, it was not possible with their study design to conclude whether there was a direct effect on measured endpoint s or whether the observed effects on the endpoints were secondary to an effect on the immune system or metabolic changes resulting from elevated cortisol levels. Moreover, the authors never clearly demonstrate that the stress response is due the addition of the formulation as opposed to other factors including the environment and the culture conditions. It is also important to recognize that spawning was experimentally manipulated with a pituitary extract which is not relevant to natural populations in the field and the lack of valid replication.

The authors speculate that the lower number of swim-up fry may have resulted from either lower fertilization rates (note: semen was taken from unexposed males), egg viability, hatching rates, embryo survival or development. The authors have not linked the observed effect on swim-up production to a mechanism of action related to an endocrine mechanism. Lower E2 levels only after 40 days of exposure may reflect a general stress response resulting from 40 days of exposure to 50% of the 96-hr LC50 value. Consistent with this general stress hypothesis is the higher liver-somatic index measured in the treatment group, which is a classic stress response and is an outcome elevated cortisol levels.

Consequently, the strength of the evidence for lowered E2 and a lower number of swim-up fry occurring through a specific endocrine mechanism (not related to a stress response) is judged to be weak.

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Author(s)	Year	Study title
Tierney, K. B., Ross, P. S., Jarrard, H. E., Delaney, K. R., Kennedy, C. J.	2006	Changes in juvenile coho salmon electro-olfactogram during and after short-term exposure to current-use pesticides. Environmental Toxicology and Chemistry Volume: 25 Issue: 10 Pages: 2809-2817 Url: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17022425 DOI: 10.1897/05-629R1.1 ISSN: 0730-7268 (Print)

Abstract[15]

For anadromous salmonids, olfaction is a critical sense, enabling return migration. In recent years, several pesticides have been identified that interfere with salmonid olfaction at concentrations in the µg/L range; thus, they may pose a risk to species longevity. In the present study, we investigated the acute effects of five agricultural pesticides on juvenile coho salmon *(Oncorhynchus kisutch)* olfaction using the electro-olfactogram (EOG), a measure of odorant-evoked field potentials. Electro-olfactogram responses to the odorant L-serine were measured during and following a 30-min exposure of the left olfactory rosette to chlorothalonil, endosulfan, glyphosate acid, iodocarb (IPBC), trifluralin, and 2,4-dichlorophenoxyacetic acid. With the relatively insoluble pesticides endosulfan and trifluralin, decreases in EOG amplitude were only apparent at relatively high concentrations (100 and 300 µg/L, respectively) following 20 min of exposure and were absent for chlorothalonil (1 mg/L). With the water-soluble herbicide glyphosate, significant EOG reductions occurred within 10 min of exposure to 1 mg/L and more rapidly with higher concentrations. Recovery of EOG post-glyphosate exposure was concentration-dependent, and complete recovery was not observed with some concentrations at 60 min postexposure. Dichlorophenoxyacetic acid only affected EOG at high concentration (100 mg/L), where it eliminated EOG within 2 min of exposure. With IPBC, EOG was decreased at 25 min of exposure to 1 µg/L; higher concentrations caused decreases to occur more rapidly. Excluding IPBC and glyphosate, all EOG reductions occurred at concentrations greater than the current Canadian water-quality guidelines and reported 96-h lethality values. Our results show that olfactory neurons can be impaired rapidly by some current-use pesticides, even at exposures in the low-µg/L range.

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate acid
Active substance(s):	Glyphosate acid
Adjuvant:	Not stated/not determined
Description:	Not stated
Source of test substance:	Sigma Aldrich, Oakville, ON, Canada
Lot/Batch #:	Not stated
Purity:	99%
Stock solution:	None, test item was added directly to test medium

2. Vehicle and/or positive control: None

[15] Quoted from article

3. Test organism:

Species:	Coho salmon (*Oncorhynchus kisutch*)
Age of test organisms at study initiation:	Parr (juveniles > 1year old)
Size of test organisms at study initiation:	16.5 ± 0.86 g, 11.4 ± 0.25 cm, condition factor (fork length): K_{fl} = 0.971 ± 0.021
Source:	Fisheries and Oceans Canada Capilano Hatchery (North Vancouver, BC, Canada)
Holding conditions prior to test:	Maintained indoor in 700 L tanks supplied with filtered, dechlorinated municipal water.
Acclimatisation:	Fish were acclimated until variation between pre-exposure EOGs determined 5 min. apart was 5% or less.

4. Test system:

Study type:	*Ex vivo*
Guideline:	None.
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	60 min.
Test conditions:	Following acclimatisation to the apparatus, fish were anaesthetised using 2-phenoxyethanol. Fish were then placed in Plexiglas holder and gills were continuously perfused with test medium and anaesthetic. The olfactory rosette of the left naris was irrigated with test medium only and test medium containing treatment mixtures (flow rate ~1.5 mL/min.). Specimens were exposed for 2, 5, 10, 15, 20, 25 and 30 min to the test substance followed by line washout following for 30 sec and a post exposure period of 2, 5, 10, 15, 20, 30, 40, 50, and 60 min.
Test concentrations:	0.1, 1.0, 10 and 100 mg/L (nominal),
Replicates per concentration:	6 for control and treatment groups
Organisms per replicate:	1
Feeding:	*Ad libitum* with commercial salmon pellets (EWOS, Surrey, BC, Canada)
Parameters measured:	Heart rates were monitored constantly during exposure. Electro-olfactograms (EOS) were recorded following delivery of 2-s pulses of 10^{-3}M of stimulus to the nasal background water flow using a computer-controlled, solenoid-valve system. Direct current EOGs were amplified 1000-fold and digitized at 200 Hz. Single serine evoked EOGs were recorded at 2, 5, 10, 15, 20, 25 and 30 min during exposure and at 2, 5, 10, 15, 20, 30, 40, 50, and 60 min. post exposure. Differences between control groups (were tested using a two-way (time and treatment), repeated- measures analysis of variance followed by a Holm-Sidak post hoc test. Median inhibitory concentration (IC_{50}) was determined for each exposure time by fitting curves to EOG inhibition versus glyphosate concentration and interpolating 50% EOG reduction. For simplicity, a three-parameter exponential decay model (inhibition = $y_o + a \cdot e^{b[glyphosate]}$) was used. 95%

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confidence intervals (CIs) were established fitting curves to twofold the standard error above and below each point. To view interactions in IC_{50} and exposure duration, the IC_{50} was plotted against exposure time, and best-fit models were used. To model the time required to cause an EOG decrease, the time at which a significant drop in EOG occurred was plotted against pesticide concentration, and curves were fitted. Additionally, EOG changes at the beginning and end of exposure and recovery periods were plotted.

Analytical determination of test concentrations: Not measured

5. Environmental conditions: (refers to holding conditions prior to lab testing)

Test medium: Filtered, dechlorinated municipal water.

Temperature: Approx. 10 °C

Photoperiod: 12 h light with 30 min transition period

Light intensity: Not stated

pH: 6.8

Oxygen saturation >90%

Conductivity: Not stated

Hardness: 6.12 mg $CaCO_3$/L

Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Test parameters do not comply with a specific test guideline, but are well documented.

2. Relevance of study: **Not relevant in the context of field exposure levels**

Comment: Significant electro-olfactogram (EOG) decreases occurred more rapidly with increasing concentration of glyphosate. Recovery after 100 mg/L was time-dependent.
By 60 min post exposure, 1 and 100 mg/L exposures remained significantly different from control, at 74.5 ± 11.7% and 72.8 ± 11.0%, respectively, of their pre-exposure EOG. After 2 min of exposure, the IC_{50} was 10.9 mg/L, and at 5 min, it dropped to 8.17 mg/L and then remained at a similar level for the remainder of the exposure. It should be recognized that the lowest effect concentration measured in this study was 1 mg a.s./L which is approximately 1000 times the Tier 1PEC_{sw} from FOCUS modelling.

3. Klimisch code: **Klimisch rating of 3**

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Amphibian Toxicity

Since the 2001 EU glyphosate evaluation, a number of acute and chronic amphibian toxicity studies with glyphosate and commercial glyphosate-based formulations have been published. For this review of the literature, studies have been divided into acute and chronic with acute studies considered to be 96 hours or less. Most of the acute and chronic amphibian studies are from laboratory toxicity tests. However, some of the studies were performed in the field using *in situ* enclosures or field mesocosms to assess impacts under representative natural conditions, and chemical and biological monitoring studies conducted under conditions directly relevant to product use. Studies were evaluated based on criteria of reliability and relevance/adequacy or risk assessment. The relevance of these studies to the glyphosate re-evaluation was primarily judged by considering the formulation that was tested and the levels and duration of exposure. The relevance of a study if it was not performed with the lead formulation was an additional consideration as well as was the study performed using environmentally relevant levels/durations of exposure.

Acute amphibian studies

A significant amount of research has been reported on the amphibian toxicity of glyphosate and several glyphosate-based formulations. Acute studies with glyphosate acid and glyphosate IPA demonstrate low toxicity. Values obtained for sensitive Gosner stage 25 tadpoles are comparable to values obtained with fish in regulatory studies and in the literature (Table 1 below and Table section IIA 8.2.1 1). The LC_{50} values range from >17.9 to >466 mg a.s./L and provide large margins of safety when compared with the PEC_{sw} from FOCUS step 1 of 0.101 mg a.s./L.

Table 1. Toxicity of glyphosate acid and IPA salt to amphibians

Test substance	Species	Duration (h)	LC_{50} mg/L	Reference
Glyphosate acid	*Crinia insignifera*, tadpole	96	103.2	Bidwell and Gorrie 1995
Glyphosate acid	*Crinia insignifera*, adult	96	75	Bidwell and Gorrie 1995
Glyphosate acid	*Litoria moorei*, tadpoles	48	81.2	Mann and Bidwell 1999
Glyphosate acid	*Litoria moorei*, tadpoles	48	121	Mann and Bidwell 1999
Glyphosate acid	*Crinia insignifera*, adult	48	83.6	Mann and Bidwell 1999
Glyphosate IPA	*Rana clamitans*	96	>17.9[1]	Howe et al., 2004
Glyphosate IPA	*Lymnodynastes dorsalis*, tadpoles	48	>400	Mann and Bidwell 1999
Glyphosate IPA	*Litoria moorei*, tadpoles	48	>343	Mann and Bidwell 1999
Glyphosate IPA	*Crinia insignifera*, tadpole	48	>466	Mann and Bidwell 1999
Glyphosate IPA	*Heleioporus eyrei*, tadpole	48	>373	Mann and Bidwell 1999

[1] This represents the highest concentration tested and no mortality was observed at this concentration.

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In total, the effects of approximately one dozen different glyphosate-based formulations have been evaluated on almost 30 species of amphibians. As previously stated, the range LC_{50} values reported for tadpoles is comparable to the range of LC_{50} values reported for fish making fish an appropriate surrogate for tadpoles. There is a mechanistic explanation why fish and tadpoles have very similar sensitivities to the surfactants that are added to glyphosate-based formulations. The toxic mode of action of surfactants to aquatic organisms is nonspecific and characterized by increasing the permeability of cell membranes resulting loss of osmotic or ionic stability at the gill. Consequently, the mode of action of surfactants to aquatic organisms is the underlying principle that explains why the range of sensitivities for amphibians and fish are remarkably similar when exposed to comparable glyphosate-based formulations (Abel and Skidmore, 1975).

With an understanding that acute toxicity data for fish is a reasonable surrogate for tadpoles, the potential for acute toxicity of MON 52276 to tadpoles can be evaluated using existing data endpoints from acute fish studies. The 96 hour LC_{50} values for MON 52276 with rainbow trout and with common carp were >306 mg a.s./L (>989 mg/L MON 52276) and >277 mg a.s./L (>895 mg/L MON 52276), respectively (from Table 10.2-1). These LC_{50} values, in the hundreds of parts per million, provide very large margins of safety when compared with the PEC_{sw} from FOCUS step 1 of 0.101 mg a.s./L. It is important to point out that uses for MON 52276 does not include direct overspray of water and consequently aquatic exposures will result from a combination of runoff and spray drift[16]. However even in cases of direct overspray in a shallow water body (e.g., 30 cm) there will be a large margin of safety even at the maximum rate of 2.88 kg a.s./ha [17]. Based on the low toxicity of glyphosate acid, glyphosate salts and MON 52276 to aquatic organisms, there is a very low likelihood of acute effects to amphibians in under field exposure conditions.

A number of studies on toxicity of glyphosate-based formulations to amphibians have been published by the Relyea lab. However, these studies are not relevant to assess whether the actual agricultural use of glyphosate and glyphosate-based formulations can harm frog populations. The concentrations in water that purportedly cause lethality to tadpoles are at least an order of magnitude higher than what is found in the environment, even after an overspray (Battaglin et al. 2009; Goldsborough and Beck 1989; Goldsborough and Brown 1993; Newton et al. 1984; Scribner et al. 2007, Tsui and Chan 2008). Thus, the extrapolation of observations from a lab study to the environment is not supported by simply examining known concentrations. Furthermore, even when caged frogs were tested during actual commercial applications, no unusual adverse effects were noted, either following exposure to expected environmental concentrations (Wojtaszek et al. 2004; Edge et al., 2011) or residues resulting from commercial forestry spray operations (Thompson et al. 2004). A deterministic and probabilistic risk assessment applied to direct oversprays of glyphosate-containing herbicides also concluded little impact on a diversity of aquatic species (Solomon and Thompson 2003).

Shortly after the Relyea papers were published RIVM in the Netherlands reviewed these studies and put them into the context of realistic environmental exposures and standard ecological risk assessment. This review by Dr. Robert Luttik is pasted below and key points were as follows:

Conclusions

- The product Roundup and other products containing the surfactant polyethoxylated tallowamine (POEA) are acutely toxic to frogs.
- Several formulations are more toxic to aquatic organisms than would be expected based on the data for non-formulated glyphosate.
- The toxicity to frogs and water fleas of the surfactant POEA by itself is of the same order of magnitude as the toxicity of formulations containing this surfactant.
- The various formulations of glyphosate differ greatly in their individual toxicities.

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[16] Considering a maximum application rate of 8 L/hectare (2.88 kg a.s./Ha), a formulation density of 1.172 g/mL, and drift from a distance of 1 m from the edge of the field (2.77% of applied) into a 30 cm deep water body, the maximum concentration of formulation that would be expected to be found in the water body is 0.0864 mg formulation/L.

[17] TER_a = (>895 mg /L MON 52276) / (3.1 mg/L MON 52276) = >288

- The above makes it reasonable to assume that the effects of the formulations are at least in part due to surfactants and/or other components.
- The sensitivity of frogs is of the same order of magnitude as the sensitivity of the organisms used for authorisation assessments in the Netherlands (algae, Lemna, water fleas and fish).
- The toxicity to algae, water fleas and fish of Roundup Evolution, the only product authorized for use on paved surfaces, is lower than the toxicity of products known to contain POEA. Although no data regarding frogs is available for this product, it is reasonable to assume that this also applies to frogs.
- **Based on the above, it is concluded that there is no reason to suppose that frogs are inadequately protected by the authorisation standards of the Netherlands.**

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Glyphosate
Advice regarding possible risks for amphibians
C.E. Smit

22-04-2005

Assignment information

SEC report number	09929a00
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Client	J. de Rijk, VROM
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Author(s)	C.E. Smit
Reviewer	**R. Luttik**; date reviewed: 19-04-2005
Assignment	Advice on risks of glyphosate for amphibians

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Conclusions

- The product Roundup and other products containing the surfactant polyethoxylated tallowamine (POEA) are acutely toxic to frogs.
- Several formulations are more toxic to aquatic organisms than would be expected based on the data for non-formulated glyphosate.
- The toxicity to frogs and water fleas of the surfactant POEA by itself is of the same order of magnitude as the toxicity of formulations containing this surfactant.
- The various formulations of glyphosate differ greatly in their individual toxicities.
- The above makes it reasonable to assume that the effects of the formulations are at least in part due to surfactants and/or other components.
- The sensitivity of frogs is of the same order of magnitude as the sensitivity of the organisms used for authorisation assessments in the Netherlands (algae, Lemna, water fleas and fish).
- The toxicity to algae, water fleas and fish of Roundup Evolution, the only product authorized for use on paved surfaces, is lower than the toxicity of products known to contain POEA. Although no data regarding frogs is available for this product, it is reasonable to assume that this also applies to frogs.
- **Based on the above, it is concluded that there is no reason to suppose that frogs are inadequately protected by the authorisation standards of the Netherlands.**

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Does glyphosate pose a risk to amphibians?

Introduction
Reports have appeared in the press to the effect that Roundup, a herbicide based on glyphosate, is highly toxic to frogs. These reports were triggered by publication of a study by Professor Rick Relyea of the University of Pittsburgh, Pennsylvania, USA (see http://www.pitt.edu/~relyea/).

The question is whether this study gives cause to suppose that the risk to frogs and other amphibians is inadequately covered by the authorisation process of the Netherlands for products based on glyphosate. The following report deals with this question in some detail. First, the article is discussed briefly. Next, a summary other recent data on the toxicity of glyphosate and products containing glyphosate to frogs is provided, and a comparison is made with the data used for environmental assessment in the Netherlands.

Summary of the article by Relyea

The article ‘The lethal impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities’ (*Ecological Applications*, 15:618–627) reports on an experiment in which an aquatic community was exposed to Sevin (carbaryl), malathion, 2-4D, or Roundup (glyphosate). This was what is called a microcosm study, in which 25 species belonging to different functional groups, predators, herbivores and zooplankton, were exposed for two weeks in 1000-litre tanks. The concentrations used in the study were 0.51 mg carbaryl/L, 0.32 mg malathion/L, 0.12 mg 2-4D/L, and 3.8 mg glyphosate/L. At the end of the exposure period, the numbers of surviving animals and their total biomass were determined and the primary production was measured in the form of periphyton (algae).

As expected, the *insecticides* caused a decline in the abundance and diversity of the predators (primarily insects), although some species did not experience any effect. Among the zooplankton species, the cladocerans (water fleas) disappeared almost completely, which led to an increase in the abundance of copepods (‘oar-foot’ crustaceans). Algae growth increased as an indirect effect of this shift. There was no adverse effect on the large herbivores (snails and tadpoles), and the survival rate of tadpoles actually increased, probably as an indirect result of a decline in predation by insects.

The herbicide 2-4D had almost no effect on any of the groups, and the biomass of the algae was also not reduced.

In the tanks treated with Roundup, 100% mortality occurred in the frog species *Rana pipiens* (leopard frog) and *Hyla versicolor* (gray tree frog), and a third species, *Rana sylvatica* (wood frog), was practically exterminated. It must be noted that the survival of these three species in the control group was not high (25–50%). There were no significant effects on two other species – *Bufo americanus* (American toad) and *Pseudacris crucifer* (spring peeper). The abundance of predators also declined, probably as an indirect result of the loss of prey animals. The biomass of algae increased. Together with the fact that a large part of the frog mortality occurred within 24 hours, this indicates that Roundup had a direct effect on the tadpoles.

Based on a comparison with toxicity data from studies of the active substance glyphosate alone and studies of formulations, the author assumed that the toxicity of Roundup was due to the surfactant (polyethoxylated tallowamine or POEA, MON 0818).

Several remarks can be made with regard to this study. In particular, it is questionable whether the systems were adequately in balance before the start of the experiment. The survival of several of the introduced species in the control group was low. Furthermore, the duration of the experiment (two weeks) was too short to allow any conclusions to be drawn regarding long-term effects at the community or ecosystem level. However, it is possible to draw the following conclusion:

- **The product Roundup caused acute mortality in several species of frogs at a concentration of 3/8 mg a.s./L.**

In a response, Monsanto expressed several objections to this study
(see ttp://www.monsanto.co.uk/news/ukshowlib.phtml?uid=8800).
These objections focused on the following points:

- the concentration that was used was not realistic;
- the absence of sediment in the system was not realistic;
- the results were inconsistent with other studies where a smaller effect or no effect was observed;
- the conclusion that the surfactant was toxic was not based on a study of the surfactant by itself.

The researcher generated a response to all of these points (see http://www.pitt.edu/~relyea/Roundup.html).

The primary issue of this discussion is whether the study presents a good picture of the risks of Roundup under field conditions. Although this discussion is inherently useful, a different approach has been taken to answer the question of whether amphibians are adequately protected based on the risk assessments performed in the Netherlands. This is because the most important questions are actually:

- whether frogs are more sensitive to Roundup and/or glyphosate than the species used in authorisation assessments in the Netherlands;

- whether there is in fact reason to assume that the effect is due to the formulation instead of the active substance, as this would mean that the toxicity of Roundup to frogs would not be directly applicable to formulations having a different composition.

The latter point is already included in the authorisation assessment with respect to algae, crustaceans and fish, for which the data of the formulation concerned are used to the extent possible to determine the acute toxicity (see http://ctb.agro.nl.ctb_files/11228_09.html).

Comparison of toxicity data

A summary of recent data regarding the toxicity of Roundup to frogs was prepared, based on the literature cited by Relyea. The original articles were consulted for some of the citations, while other data were taken from the text without further evaluation. The data were then compared with the toxicity data for other species used in the Netherlands risk assessment, which can be found on the CTB website
(see http://ctb.agro.nl.ctb_files/11228_09.html).

A summary of the data is provided in Annex 1. The products authorized for use in the Netherlands are listed in Annex 2. As the compositions of these products are not public, POEA is only indicated as being present in the formulations listed in Annex 1 for those products for which this information is known from published literature.

From the comparison, it can be seen that:

- a number of formulations are more toxic than would be expected based on the data for non-formulated glyphosate, but this does not apply to Roundup Dry, Roundup Evolution or Rodeo;
- the formulations differ in toxicity;
- the toxicity to frogs and water fleas of the surfactant POEA by itself is of the same order of magnitude as the toxicity of formulations containing this surfactant;
- based on the above, it is reasonable to assume that surfactants and/or other components are a significant factor;
- the sensitivity of frogs is of the same order of magnitude as the sensitivity of species that are used in the Netherlands for authorisation assessments (algae, Lemna, water fleas and fish);
- the toxicity to algae, water fleas and fish of Roundup Evolution, the only product authorized for use on paved surfaces, is lower than the toxicity of products that are known to contain POEA. Although no data regarding frogs is available for this product, it is reasonable to assume that this also applies to frogs.

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Conclusion

Based on the above, it is concluded that there is no reason to suppose that frogs are inadequately protected by the authorisation standards of the Netherlands.

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Annex 1: Summary of available toxicity data

FROGS	Glyphosate and glyphosate isopropylamine salt	Acute LC50	108 – >450	mg a.s/L
	formulation without POEA (Roundup)	Acute LC50	9729	mg a.s/L
	formulations with POEA	Acute LC50	0.88 – 15.5	mg a.s/L
	(Glyfos,[a] Roundup,[a] Vision)	Chronic LC50	0.6 – 2.5	mg a.s/L
		Chronic NOEC	1 –2	mg a.s/L
	POEA alone	Acute LC50	6.8	mg/L
ALGAE	Glyphosate	EC50	1.30 – 48	mg a.s/L
	Roundup (with POEA)[a]	EC50	0.66 – >75.6	mg a.s/L
	Roundup Dry[b]	EC50	149	mg a.s/L
	Roundup Evolution[a]	EC50	55 – 88	mg a.s/L
	Roundup TX[b]	EC50	0.002 – 0.033	mg a.s/L
	Luxan glyphosate liquid[a]	EC50	2.79	mg a.s/L
	Agrichem glyphosate B	EC50	1.80	mg a.s/L
	Glyfos[a] (with POEA)	EC50	16.2	mg a.s/L
LEMNA	Roundup[a] (with POEA)		23.4	mg a.s/L
WATER FLEAS	Glyphosate	Acute EC50	>100 – 780	mg a.s/L
	Roundup[a] (with POEA)	Acute EC50	1.6 – 59.4	mg a.s/L
	Roundup Dry[b]	Acute EC50	>405	mg a.s/L
	Roundup Evolution[a]	Acute EC50	208	mg a.s/L
	Roundup TX[b]	Acute EC50	1.6	mg a.s/L
	Glyphosate 360	Acute EC50	6.9	mg a.s/L
	Luxan glyphosate liquid[a]	Acute EC50	3.4	mg a.s/L
	Agrichem glyphosate B	Acute EC50	3.8	mg a.s/L
	Glyfos[a] (with POEA)	Acute EC50	6.9	mg a.s/L
	POEA alone	Acute EC50	2.0	mg/L
	Glyphosate	Chronic NOEC	9.4 – 100	mg a.s/L
	Roundup (with POEA)	Chronic NOEC	0.99	mg a.s/L
	Roundup TX[b]	Chronic NOEC	0.17	mg a.s/L
	Agrichem glyphosate B	Chronic NOEC	0.28	mg a.s/L
	Glyfos[a] (with POEA)	Chronic NOEC	0.47	mg a.s/L
FISH	Glyphosate	Acute EC50	86 – >1000	mg a.s/L
	Roundup[a] (with POEA)	Acute LC50	3.4 – 4.3	mg a.s/L
	Roundup Dry[b]	Acute LC50	>393	mg a.s/L
	Roundup Evolution[a]	Acute LC50	>306	mg a.s/L
	Roundup TX[b]	Acute LC50	0.7	mg a.s/L
	Glyphosate 360	Acute LC50	3.71 – 5.67	mg a.s/L
	Sting 306	Acute LC50	0.81 – 1.35	mg a.s/L
	Luxan glyphosate liquid[a]	Acute LC50	4.2	mg a.s/L
	Agrichem glyphosate B	Acute LC50	4.2	mg a.s/L
	Glyfos[a] (with POEA)	Acute LC50	3.7	mg a.s/L
	Glyphosate	Chronic NOEC	25.7 – 52	mg a.s/L
	Roundup (with POEA)[a]	Chronic NOEC	0.74	mg a.s/L
	Roundup TX[b]	Chronic NOEC	0.13	mg a.s/L
	Glyphosate 360	Chronic NOEC	0.15	mg a.s/L
	Agrichem glyphosate B	Chronic NOEC	0.14	mg a.s/L
	Glyfos[a] (with POEA)	Chronic NOEC	0.25	mg a.s/L

a: Authorized products
b: Discontinued products
The other products are not shown on the list of authorized products (see Annex 2), although some of them may actually be authorized under other names.

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Annex 2: Summary of glyphosate-based products authorized for use in the Netherlands

Product	Authorisation number
Agrichem glyphosate	7866
Agrichem glyphosate 2	10945
Agrichem glyphosate B	10946
Amega	12661
Clear-Up 360	12383
Clear-Up P	12593
Clinic	11962
Envision	12523
Glifonex	11040
Glycar	11055
Glyfall	11676
Glyfos	11227
Glyfos Envision 120 g/L	12594
Glyfos Envision 7.2 g/L	12595
Glyper 360 SL	12216
Glyphogan	11230
Greenfix	11628
Holland Fyto Glyfosaat	10262
Imex-glyfosaat 2	8597
Klaverblad-glyfosaat	10045
Luxan glyphosate liquid	10793
Onkruid totaal	11976
Onkruiddoder	12634
Panic	12639
Roundup	6483
Roundup Econ 400	11553
Roundup Energy	12546
Roundup Evolution	11228
Roundup Huis & Tuin	10099
Roundup Max	12545
Roundup Ready to Use	10867
Sphinx	11041
Touchdown Quattro	12552

The only product authorized for use on paved surfaces is Roundup Evolution.

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Chronic amphibian studies:

Several laboratory chronic exposure studies have been performed that have looked at growth, development and survival. Adverse chronic effects of glyphosate acid and glyphosate salts have not been reported. Chronic effects have only been reported in formulation studies where tadpoles were exposed to formulation levels that would only be observed after directly over spraying a shallow water body. The potential for surfactants to cause adverse effects to aquatic organisms should only be evaluated in the context of an environmentally realistic exposure. Therefore, the processes of surfactant dissipation from the water column, biodegradation, and bioavailability must each be considered to evaluate the potential for risk. Studies that have investigated the relationship between acute aquatic toxicity and biodegradation with alkylamine ethoxylates have demonstrated that loss of toxicity correlates with biodegradation (van Ginkel et al. 1993a; van Ginkel et al. 1993b). Therefore, surfactant-induced histopathological effects observed in laboratory experiments conducted under continuous exposure must be evaluated with caution since the environmental fate of the compound has not been included in the exposure regime.

Since the last glyphosate evaluation, a study was performed that examined the dissipation of POEA as a representative surfactant in a water sediment system (Wang et al. 2005). In this study, the POEA surfactant MON 0818 was prepared at a target concentration of 8 mg/L in well water. Two natural sediments (1.5% and 3.0 % organic matter) were layered into triplicate separate glass aquaria (80 x 30 x 30 cm) to a depth of 3 cm and then 12 cm of the MON 0818-containing water was added to the sediments or to empty aquaria. Well water alone was also added to each of the sediments as another treatment in the study. The removal of MON 0818 from the water column was monitored over time using both a biological assay and chemical analysis. Water samples were removed from each aquarium at 2, 6, 24, 48, 72, and 96 hours. The samples were analyzed using a toxicity bioassay with Daphnia magna and using high performance liquid chromatography with mass selective detection for intact surfactant. The authors reported, "The toxicity of the POEA surfactant, MON 0818, decreased rapidly in water from microcosms containing sediment, and this decrease in toxicity was correlated with the decline of MON 0818 concentrations in the overlying water." Dissipation rates could be calculated from the analytical measurements of POEA over time, and DT50 values for the removal of POEA from the water column in the presence of the two sediments were calculated to be 18 hrs and 13 hrs, respectively. It is likely that both physical adsorption to the sediment and microbial degradation played some role in the rapid reduction in toxicity and surfactant removal from the water column. However, since the instrumental analysis only focused on the molecular ions for the intact surfactant molecules, no estimate of the contribution of microbial degradation to the dissipation rate can be made from this study.

A recent study that examined the acute toxicity of two glyphosate-based formulations demonstrated a significant decrease in acute toxicity by improving the realism of the acute exposures by adding sediment to the experimental tanks (Fuentes, 2008). Thus, the extrapolation of observations from a lab study to the environment is not supported by simply predicting worst-case field exposures and not taking dissipation and degradation of the formulation components into consideration. Below is a summary of comparisons made using the original formulation of Roundup (MON 2139).

Responses of six larval anuran species to the original formulation of Roundup® (MON 2139) in aqueous and water-sediment exposures.[1]

Species Tested	Sediment Present	96h LC_{50} (mg a.s./L)	NOEC
R. pipiens	No	1.80 (1.73-1.88)	1.29
R. pipiens	Yes	4.83 (4.83-5.16)	2.96
R. sphenocephala	No	2.05 (1.90-2.20)	1.52
R. sphenocephala	Yes	5.13 (4.87-5.41)	3.53
H. chrysoscelis	No	2.50 (2.38-2.63)	1.74
H. chrysoscelis	Yes	4.37 (4.17-4.62)	2.76
R. catesbeiana	No	2.77 (2.66-2.89)	2.02

R. catesbeiana	Yes	6.09 (5.83-6.40)	4.84
B. fowleri	No	4.21 (4.08-4.33)	3.40
B. fowleri	Yes	5.84 (5.59-6.10)	4.41
R. clamitans	No	4.22 (4.02-4.42)	3.27
R. clamitans	Yes	8.26 (8.04-8.49)	5.34

[1] The original formulation of Roundup® contains ~30% glyphosate (w/w %) as a.s. and 15% MON 0818.

Below are key points to consider in the review of acute aquatic amphibian studies published in the open literature.

- Application directly to water is not is not part of the GAP.
- Interpretation of the results of laboratory or outdoor tank or mesocosm aquatic studies is always uncertain because pesticide application rates are calculated in mass per unit area (e.g. pounds per acre or kilograms per hectare) but aquatic exposures are generally expressed in mass per volume (e.g., mg per liter). Therefore, assumptions about water depth have a great deal of impact on the ensuing risk calculations. Furthermore, kinetic rates of mixing of the initial surface deposition throughout the water column, as well as the degree of contact of dissolved components of the water column with sediment either suspended or in the benthic layer, strongly influence the shape of the exposure curve during the hours and days following application. These factors, and many others, form the basis for differences between hazards that may be implied by laboratory tests versus effects that will be observed under actual exposure conditions. Dose-response curves for aquatic organism toxicity can be very steep, so that the difference between an LC_{10} and an LC_{90} concentration may differ by no more than 2- or 3-fold in some instances.
- To determine glyphosate concentrations (and by ratio, surfactant concentrations) that might result from forestry uses in the event of accidental overspray of shallow ponds or wetlands, several studies have been conducted with aerial application equipment in which glyphosate formulations have been intentionally sprayed over or adjacent to shallow ponds or wetlands at forestry sites (Goldsborough and Brown, 1993; Newton et al., 1994; Goldsborough and Beck 1989; Thompson et al., 2004). These studies demonstrate that glyphosate concentrations in shallow water rarely exceed 0.8 mg a.e./L (see Table 1 at the end of this amphibian section), and when this concentration is exceeded, it arises from a very high application rate and is observed for a very short period of time, generally less than one day.

Summary of glyphosate concentrations measured in shallow static water bodies after intentional aerial application to these water bodies

Type of Water Body	Depth (m)	Application Rate (kg Gly a.e./ha)	Maximum Initial Concentration[1] (mg Gly a.e./L)	Subsequent or Mean Concentrations (mg Gly a.e./L)	Reference
Small Ponds	0.9–1.5	2.1	0.0636 0.0837 0.0462		Goldsborough & Brown 1993
Small Ponds	<1	4.12 [3]	0.090 1.678 0.983	0.001 (Day 1) 0.307 (Day 1) 0.049 (Day 1)	Newton *et al.*, 1994
Microcosms 30x 45 x 30 cm - sediment + sediment Ponds	0.3 0.25 - 1.5	0.89	0.352 0.215 0.037 0.050 0.055 0.141	0.025 [2] 0.027 [2] 0.055 [2] 0.105 [2]	Goldsborough & Beck 1989
Wetlands Oversprayed Adjacent Buffered	0.15 – 0.80	1.92	<0.01 - 1.95[4] <0.01 - 0.74[5] <0.01 - 0.31[6]	0.33 [2] 0.18 [2] 0.03 [2]	Thompson *et al.*, 2004

[1]Highest concentration observed at each site during the study
[2]Mean concentrations
[3]This rate is indicated as three times the normal use rate.
[4]Total of 24 sites.
[5]Total of 11 sites.
[6]Total of 16 sites.

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Author(s)	Year	Study title
Brodman, R., Newman, D.W., Laurie, K., Osterfeld, S., Lenzo, N.	2010	Interaction of an Aquatic Herbicide and Predatory Salamander Density on Wetland Communities Journal: Journal of Herpetology Volume: 44 Issue: 1 Pages: 69–82 Url : http://www.bioone.org/doi/abs/10.1670/08-320.1 DOI: 10.1670/08-320.1 ISSN: 0022-1511 (print), (online)

Abstract[18]

Pesticides can be important conservation tool, but they could have unintended impacts on amphibians. The commercial glyphosate-based herbicide Accord is approved for use in wetlands and ponds because it is designed to be safer to aquatic wildlife than other herbicide formulations (e.g., Roundup or Atrazine); however, field experiments are needed to determine whether there are direct, indirect, or sublethal effects on amphibians or effects on wetland community structure. We conducted a replicated field experiment in constructed ponds to test for both the effects of Accord and predator (Tiger Salamanders, *Ambystoma tigrinum*) density on amphibians and aquatic invertebrates. Herbicide treatment had significant density-dependent effects on Tiger Salamander growth, development, and survival. The survival of anurans and aquatic invertebrates was also affected by herbicide treatment and predator density. At certain Tiger Salamander densities, the community structure was altered such that some species became more common with herbicide treatment, whereas others became less common. Behavior assays of salamander larvae suggest that herbicide treatment alters predator-prey relationships in the experimental pond communities. These results suggest that competition and predation may mediate indirect effects of this herbicide on the aquatic fauna. We conclude that exposure to Accord poses less of a risk to the ecology of amphibians than do other formulations of glyphosate-based herbicides.

MATERIALS AND METHODS

1. Test material:

Test item: 5% herbicide mixture of Accord and 3% Cide-Kick II (aquatic surfactant)

Active substance(s): Glyphosate

Surfactant: D'1-limonene, a nonylphenolpolyethylene(NPE)-based product (wetting agent Cide-Kick II ®)

Description: Not precisely stated, not clear which formulation was tested. It was stated that the formulation is approved for use in wetlands and ponds especially to control the invasive Reed Canarygrass (*Phalaris arundinacea*), no a.s. loading reported.

Source of test substance: not stated

Lot/Batch #: Not stated

Purity: not stated

2. Vehicle and/or positive control: none

3. Test organism:

Species: Experimental pond communities containing tadpoles of different species of amphibians

[18] Quoted from article

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	Ambystoma tigrinum *Rana pipiens* *Rana clamitans* *Bufo americanus* Aquatic invertebrates as natural inhabitants
Age of test organisms at study initiation:	*Ambystoma tigrinum* larvae (mesocosm study):mean size of 32.1 mm SVL[19](3 size classes "< 25 mm", "25 – 35 mm" and ">35 mm"), age not specified Laboratory assays conducted with larvae of *A. tigrinum*: not precisely stated, for microhabitat assay approximately the same age with SVL differences ranging from 5 – 10 mm
Source:	Experimental ponds of Saint Joseph´s College Biological Field Station, additional amphibians were collected at nearby wetlands within the Field Station in northwest Indiana, USA
Holding conditions prior to test:	Natural field conditions not specified further

4. Test system:

Study type:	Outdoor experimental ponds, behavioural assays were conducted *ex vivo* under laboratory conditions
Guideline:	None
GLP:	not relevant
Guideline deviations:	Not applicable
Duration of study:	Pond exposure: 18th May until end of June 2006 (ca. 1 ½ month), repeated in the year 2007 (started on 14th May, duration not stated)
Test conditions:	Pond exposure performed in experimental outdoor ponds, area: 6 x 6 m, volume: 24 m³, depth: 0.67 m Activity and feeding assays were conducted in pyrex containers (7 cm high x 20 cm wide x 20 cm long), The container was placed over a numbered grid of 15 mm wide hexes. Microhabitat assay was conducted in plastic containers (26 cm high x 23.5 cm wide x 33 cm long) filled to a depth of 16 cm with dechlorinated tap water. The containers were partitioned into two equal chambers using a plastic mesh with 1.5-cm openings. One chamber had a 2-cm layer of pondweed, leaf litter, and algae, the other chamber was left empty.
Replicates per concentration:	5
Organisms per replicate:	Pond exposure: *Ambystoma tigrinum:* 15, 30 or 47 larvae per pond *Rana pipien:* 85 tadpoles per pond *Rana clamitans:* 51 tadpoles per pond *Bufo americanus:* 11 tadpoles per pond aquatic invertebrates: natural density – based on what..? *Behaviour assay:* 6 *Activity, feeding and microhabitat assay:* 2
Feeding:	Feeding assay: one *Bufo* tadpole, 10 macroinvertebrates (mix of common species from the pond samples small enough to be potential prey for the salamander larvae), and 10 microcrustaceans (mix of Cladocerans, Copepods, and Ostracods)

[19] Snout to vent length

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Microhabitat: Small aquatic invertebrates

Parameters measured: For the activity, behaviour, feeding and microhabitat assay, samples were collected once a week and assessments were conducted in the afternoon and early evening (13:00 – 20:00h). SVL[19] of *A. tigrinum* larvae, amphibian and invertebrate density, species richness and diversity, mortality, metamorphosis, number of movements (position of the head), distance moved by the larvae, feeding activity (predation rate, prey preference), stomach content of dead larvae, behaviour (aggression, percentage of time in vegetation and time separated)

Test concentrations: 1.5 L of herbicide mixture. Concentrations were estimated to be no greater than 2.0 mg/L of active ingredient glyphosate and 1.2 mg/L of the surfactant NPE

Application: One application per year

Application devices: with hand-held sprayer

Application verification: not conducted

Analytical determination of test concentrations: not conducted

5. Environmental conditions:

Test medium: Experimental pond exposure was conducted with natural pond water, activity assay with dechlorinated tap water. Abiotic parameters (pH, alkalinity, nitrate, nitrite and dissolved oxygen) in outdoor ponds were monitored on week 1, 3, 5 and 7 but not entirely reported. No measurements for the laboratory assays are reported.

Temperature: not stated

Photoperiod: natural photoperiod depending on season

Light intensity: not stated

pH: control pond: 7.1 ± 0.5
treated pond: 7.1 ± 0.5

Dissolved Oxygen: control pond: 2.3 ± 1.8 ppm
treated pond: 3.0 ± 1.8 ppm

Alkalinity: control pond: 216.2 ± 41.9 ppm of calcium carbonate
treated pond: 215.0 ± 69.5 ppm of calcium carbonate

Nitrate: control pond: 0.9 ± 2.0 ppm
treated pond: 0.1 ± 0.2 ppm

Nitrite: control pond: 18.8 ± 34.0 ppm
treated pond: 7.1 ± 5.6 ppm

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Selection criteria for salamander or anurans are not stated.
- Information about pre-treatment of the ponds or history of pesticide application is missing.
- Experimental pond set up is unclear (source of sediments, litter, zooplankton, water, additional feeding etc.)

- Indirect effects via herbicidal effects on primary production were not considered.
- Unclear whether ponds were left for acclimatisation prior to introduction of amphibian larvae. Ponds should be left for at least 3-6 weeks to allow settling of suspended organics.
- Unclear whether specimens escaping from the exposure setups were included (refugees).
- Zooplankton densities were heavily biased towards cladoceran and copepods in the control groups across all salamander density treatments.
- Important information on the glyphosate application procedure is missing.
- Glyphosate concentrations in the mesocosms were not measured post application and throughout the study.
- Claimed glyphosate concentrations tested of 1 and 2 mg a.i./L exceed realistic environmental field exposures. Exposure concentrations likely exceeded this. It is stated that 1.5 L of the herbicide mixture (5% a.i. and 3% Cide-kick) were added to the 24,000 L mesocosms each year. Calculations indicate this volume of solution and concentration glyphosate would result in 3.125 mg a.i./L and the Cide-Kick concentration would be 1.875 mg/L.
- The laboratory assays are not well-documented and data about test conditions are incomplete.

2. Relevance of study: **Not relevant**

Comment: This study tested a mixture of glyphosate and nonylphenolpolyethylene (NPE)-based product at a rate of 3%. NPE is known to be highly toxic to aquatic organisms and the material test in not representative of the aquatic toxicity of currently registered formulations, particularly the lead formulation MON 52276. Effects of glyphosate versus the surfactant could not be separated because no treatment with glyphosate alone was included.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment**

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	Year	Study title
Cauble, K., Wagner, R.S.	2005	Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development Bulletin of Environmental Contamination and Toxicology Volume: 75 Pages: 429-435

Abstract

No abstract available.

Executive summary[20]:

Effects of chronic exposure to Roundup ® (50.2% glyphosate isopropylamine salt) were investigated at non-acute levels in a static renewal test on *Rana cascadae* larval metamorphosis and development. Seven larvae per treatment were exposed to five replicates of each treatment of 0, 1, and 2 mg glyphosate/L (nominal) and a control. Larvae were evaluated on a daily basis for 43 days for mortality, feeding behaviour, swimming activity, morphological abnormalities and behavioural alterations.

At the highest concentration tested (1.94 mg glyphosate/L, mean measured), no individuals survived until end of the exposure. Exposure to 1 mg glyphosate/L resulted in earlier metamorphosis and smaller size for Roundup ®, when compared to the control.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup ® but the specific formulation not specified
Active substance(s):	Glyphosate isopropylamine salt
Surfactant:	Not stated
Description:	none
Source of test substance:	Monsanto Company, St. Louis, MO
Lot/Batch #:	Not stated
Purity:	50.2% glyphosate isopropylamine salt
Stock solution:	Treatment solutions were diluted from 250 mL stock (50 mg/L) glyphosate formulation. All replicate treatments were mixed together in a 5 L beaker and 1 L was dispersed into treatment replicates. Dilutions were prepared in dechlorinated spring water.

2. Vehicle and/or positive control: none

3. Test organism:

Species:	*Rana cascadae*
Age of test organisms at study initiation:	2 weeks
Source:	Eggs collected from Table Mountain, Kittitas County, WA
Holding conditions prior to test:	Larvae hatched in 10 L glass tanks at 18 °C under incandescent light with 12 h photoperiod. Larvae were fed *ad libitum* with boiled organic lettuce.
Acclimatisation:	Not stated

4. Test system:

Study type:	Static renewal (7 day intervals)
Guideline:	Not stated
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	43 d chronic
Test conditions:	Larvae were exposed in pre-rinsed plastic containers (30 cm × 20 cm × 10 cm) tilted at an angle of approx.. 30° so the upper portion was dry and lower portion filled with 1 L of treatment

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[20] Compiled by DKC

	solution.
Replicates per concentration:	5
Organisms per replicate:	7
Feeding:	Not stated
Parameters measured:	Larvae were evaluated on a daily basis for 43 days for mortality (time to death), feeding behaviour (feeding or not feeding), swimming activity (high, medium, slow), morphological abnormalities (edema, lesions, bent tail) and behavioural alterations (head out of water, erupted forelimbs, erupted hind limbs, emersion from water). Dry mass after 43 days. Physico-chemical parameters: not stated. Mean dry mass was compared using Student's t-test; differences among replicates and treatments were evaluated using one-way ANOVA followed by Tukey-Kramer Multiple comparison tests with NCSS, as post-hoc.
Test concentrations:	1.0 and 2.0 mg formulation/L
Analytical determination of test concentrations:	Verified by HPLC-UV at start of exposure and after 7 days. Measured concentrations were not significantly different to nominal concentrations: initial: (0.96±0.13 (SE) and 1.94±0.13 (SE)). 7 days: : (0.94±0.13 (SE) and 1.92±0.13 (SE))
Validity criteria:	Not applicable

5. Environmental conditions:

Test medium:	Dechlorinated spring water with buffered pH.
Temperature:	Not stated
Photoperiod:	Not stated
Light intensity	Not stated
pH:	Not stated
Oxygen saturation	Not stated
Conductivity:	Not stated
Hardness:	Not stated
Alkalinity:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (few experimental details provided)

2. Relevance of study: **Not relevant**
Testing performed with an unknown formulation.

3. Klimisch code: **3 and not adequate for risk assessment**

Expanded comments

In the study performed by Cauble and Wagner *Rana cascadae* tadpoles were chronically exposed to two aqueous concentrations of an unidentified concentrated Roundup formulation over 43 days. Two weeks

after hatching (Gosner stage not reported) tadpoles were continuously exposed to concentrations of 1 and 2 mg glyphosate a.e./L along with an untreated control in a static renewal system with weekly renewals. Glyphosate concentrations were measured and mean measured levels were similar to nominal concentrations. It is cited by the authors that the 48 hour LC_{50} value for *R. cascadae* is 3.2 mg/L. Therefore, the selected chronic exposure levels represent a high proportion (33% and 66%) of the reported 48-hour LC_{50} value. Many experimental details are absent from the paper, such as control survival, the loading rate, water quality parameters, and the water temperature during the exposures, which question the quality and validity of this study.

As previously discussed for the Howe *et al.* publication, dissipation and degradation of glyphosate and the surfactant are likely occur rapidly, so that the duration of exposure would be significantly shorter, perhaps no more than 1 or 2 days following an application (Giesy et al., 2000; Wang et al., 2005). Therefore, a 43 day continuous exposure to concentrations of 1 and 2 mg a.e./L is very unrealistic, and the potential for effects on sublethal endpoints must be evaluated with extreme caution.

In the 2 mg/L treatment all tadpoles died over the 43-day exposure period, with a reported average time to death for individual tadpoles of approximately 7 days. Consequently, effects on all measured sublethal endpoints are considered secondary.

In the 1 mg/L treatment the average time to death was reported to be approximately 34 days within the 43-day exposure period. Therefore, it appears that the majority of mortality occurred before the end of the exposure period. Consequently, effects on all measured sublethal endpoints are considered secondary. Oddly, the mean time to death for the controls in this study are shown as approximately 52 days, although the exposure was only 43 days and control mortality is not reported.

Accelerated metamorphosis observed in the 1 mg/L treatment was consistent with a generalized stress response, rather than a specific MoA for endocrine disruption (Denver, 1997a). Tadpole metamorphosis can be induced by stress under natural conditions, such as pond drying and/or crowding, and will accelerate metamorphosis as an adaptive stress response (Denver, 1997b). This response is mediated through a cascade of signaling events, beginning with the hypothalamic secretion of corticotropin-releasing hormone which in turn stimulates the pituitary to secrete both thyroid-stimulating hormone and adrenocorticotropic hormone, which stimulate the thyroid gland to secrete thyroid hormones and the interrenal gland to secrete corticosterone, respectively (Denver, 1997a, b). Exogenously applied corticosteroids also accelerate metamorphosis in some species of amphibians although this may be stage-specific (Hayes, 1995; Hayes, 1997; Hayes et al., 1993). Therefore, there is evidence that environmental contaminants can similarly affect metamorphosis by inducing a stress response. For example, some studies have demonstrated precocious metamorphosis among tadpoles exposed to pesticides that have no known thyroid activity (Boone et al., 2001; Boone and Semlitsch, 2002; Boone and Bridges, 2003; Greulich and Pflugmacher, 2003; Rohr et al., 2004; Forson and Storfer, 2006). Consequently, the paper published by Cauble and Wagner, where there was very high treatment mortality over the course of the exposure, should be considered as an example where preconscious metamorphosis was observed as a result of extreme stress.

References:

Boone, M., Bridges, C.M., Rothermel, B.B. 2001. Growth and development of larval green frogs (*Rana clamitans*) exposed to multiple doses of an insecticide. Oecologia 129, 518–524.

Boone, M.D., Semlitsch, R.D. 2002. Interactions of an insecticide with competition and pond drying in amphibian communities. Ecological Applications 12, 307–316.

Boone, M.D., Bridges, C.M. 2003. Effects of carbaryl on green frog (*Rana clamitans*) tadpoles: timing of exposure versus multiple exposures. Environmental Toxicology and Chemistry 22, 2695–2702.

Denver, R.J. 1997a. Environmental stress as a developmental cue: corticotrophin releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Hormones and Behavior 31, 169–179.

Denver, R.J. 1997b. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. American Zoologist 37:172-184.

Forson, D., Storfer, A. 2006. Effects of atrazine and iridovirus infection on survival and life-history traits of the long-toed salamander (*Ambystoma macrodactylum*). Environmental Toxicology and Chemistry 25, 168–173.

Giesy JP, Dobson S, Solomon KR. (2000) Ecotoxicological Risk Assessment for Roundup® Herbicide. Reviews of Environmental Contamination and Toxicology 167: 35-120.

Greulich, K., Pflugmacher, S. 2004. Uptake and effects on detoxification enzymes of cypermethrin in embryos and tadpoles of amphibians. Archives of Environmental Contamination and Toxicology. 47, 489–495.

Hayes, T., Chan, R., Licht, P. 1993. Interactions of temperature and steroids on larval growth development and metamorphosis in a toad Bufo boreas. Journal of Experimental Zoology 266, 206–215.

Hayes, T.B. 1995. Interdependence of corticosterone-hormones and thyroidhormones in larval toads (*Bufo boreas*) .1. Thyroid hormone-dependent and hormone-independent effects of corticosterone on growth and development. Journal of Experimental Zoology 271, 95–102.

Hayes, T.B. 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. American Zoologist 37, 185–194.

Rohr, J.R., Elskus, A.A., Shepherd, B.S., Crowley, P.H., McCarthy, T.M., Niedzwiecki, J.H., Sager, T., Sih, A., Palmer, B.D. 2004. Multiple stressors and salamanders: effects of an herbicide, food limitation, and hydroperiod. Ecological Applications 14, 1028–1040.

Wang N, Besser JM, Buckler DR, Honegger JL, Ingersoll CG, Johnson BT, Kurtzweil ML, MacGregor J, McKee MJ. 2005. Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms. Chemosphere 59: 545–551.

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Authors	Year	Study title
Comstock, B.A., Sprinkle, S.L., Smith G.R.	2007	Acute Toxic Effects of Round-Up Herbicide on Wood Frog Tadpoles (*Rana sylvatica*) Journal of Freshwater Ecology Volume: 23 Isssue: 4 Pages: 823-831

Abstract[21]

We examine the toxicity of a commercial formulation of Round-Up to wood frog (*Rana sylvatica*) tadpoles. All tadpoles exposed to nominal concentrations ≥ 0.00098% glyphosate died within 24 h. However, tadpoles exposed to concentrations ≤ 0.00049% showed high survivorship. Our results suggest that any direct application of this formulation of Round-Up to aquatic ecosystems could have an impact on amphibian larvae, especially in shallow water.

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MATERIALS AND METHODS

1. Test material:

Test item(s): Round-Up® Weed and Grass Killer Concentrate Plus
Active substance(s): Glyphosate isopropylamine salt
Surfactant: unknown
Description:
Source of test substance: Monsanto Corporation, St. Louis, Missouri, USA
Lot/Batch #: Not stated
Purity: 18% glyphosate, 0.73% diquat dibromide
Stock solution: Not stated

2. Vehicle and/or positive control: none

3. Test organism:

Species: *Rana sylvatica* (Anura, Ranidae)
Age of test organisms at study initiation: tadpoles at Gosner stage 26
Source: Egg masses collected from local pond (Denison University, Grainville, Ohio, USA)
Holding conditions prior to test: Hatched in laboratory and maintained in large plastic containers in aged tap water until experiment.
Acclimatisation: Not stated

4. Test system:

Study type: Static
Guideline: Not stated
GLP: No

[21] Quoted from article

Guideline deviations:	Not applicable
Duration of study:	96 hours
Test conditions:	Tadpoles were exposed to different treatments in aged water in plastic containers.
Treatments:	16 glyphosate concentrations and 1 untreated control
Test concentrations:	Glyphosate nominal concentrations of 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.015625%, 0.0078%, 0.0039%, 0.00195%, 0.000977%, 0.0004885%, 0.0002425%, 0.00012425% and 0.000062125%.
Replicates per treatment:	Unknown
Organisms per replicate:	5
Feeding during experiments:	None
Parameters measured:	Mortality at 24 and 96 hours. Proportions of surviving tadpoles were arc sin transformed and compared among treatments by ANOVA.
Analytical determination of test concentrations:	None
Validity criteria:	Not applicable

5. Environmental conditions:

Test medium:	Aged tap water
Temperature:	Not stated
Photoperiod:	Not stated
Light intensity	Not stated
pH:	Not stated
Oxygen saturation	Not stated
Conductivity:	Not stated
Hardness:	Not stated
Alkalinity:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment

- This concentrated lawn and garden formulation tested contained glyphosate and diquat dibromide.
- No valid identification reported about test species.
- Insufficient information about test medium and conditions (temperature, pH, feeding, etc.)
- Number of replicates is not reported which is normally a basic parameter of every scientific study.

2. Relevance of study: **Not relevant**

Comment: Lack of analytics, unsuitable test system, poor statistical analysis and weak interpretation of data leads to an overall performance which does not allow any conclusions from study results.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment**

Author(s)	Year	Study title
Dinehart, S.K, Smith, L. M., McMurrya, S.T., Smith, P.N., Anderson, T. A., Haukos, D. A.	2010	Acute and chronic toxicity of Roundup Weathermax ® and Ignite ® 280 SL to larval *Spea multiplicata* and *S. bombifrons* from the Southern High Plains, USA Environmental Pollution *15* Volume: 158 Issue: Pages: 2610 - 2617 DOI: 10.1016/j.envpol.2010.05.006 ISSN: 0269-7491

Abstract[22]

Pesticides have been implicated in widespread amphibian declines. We assessed acute and chronic toxicity of two widely used herbicides to larval New Mexico (*Spea multiplicata*) and Plains (*S. bombifrons*) spadefoots from cropland and native grassland playas. Roundup WeatherMAX ® (WM) toxicity estimates (48- and 216-h LC_{50}; 48-h LC_1) for both species were similar to environmental concentrations expected from accidental overspray. Chronic (30-day) exposure to WM at predicted environmental concentrations (2.0 and 2.8 mg glyphosate acid equivalents/L) reduced survival of both species. Ignite ® 280 SL (IG) toxicity estimates (48-h LC_{50} and LC_1) for both species were above predicted environmental concentrations of 1.0 mg glufosinate/L. Chronic exposure to predicted environmental concentrations of IG did not reduce survival of either species. Toxicity test results suggest that at predicted environmental concentrations IG would not cause extensive mortalities among larval New Mexico and Plains spadefoots. However, WM may cause extensive mortality among larvae of these species.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Weathermax ®
Active substance(s):	Glyphosate (potassium salt form)
Adjuvant:	unknown
Description:	landuse
Source of test substance:	Commercial retail outlet, manufacturer: Monsanto Company, St. Louis, Missouri, USA
Lot/Batch #:	Not stated
Purity:	48.8 % Glyphosate, 51 % other ingredients
Stock solution:	330 g a.i./L, mixture with the formulated product and tap water

2. Vehicle and/or positive control:

3. Test organism:

Species:	Larvae of *Spea multiplicata* and *S. bombifrons*
Cultivar:	None
Source:	Southern cropland playas in Floyd, Hockley, and Terry counties (19th May) and grassland playas in Briscoe and Floyd counties (20th – 21st), Texas, USA
Age of test organisms at study initiation	Gosner stage 29 - 30 for both experimental set-ups

[22] Quoted from article

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/ Crop growth stage at treatment:

Holding conditions prior to test: Photoperiod: 14 h – 10h light-dark cycle (This photoperiod approximates that near the centre of the Southern High Plains during May - September., 21.96 ± 0.04 °C,

Medium: aerated, aged tap water, dissolved oxygen = 8.06 ± 0.08 mg/L, ammonia = 2.1 ± 0.1 mg/L. An 80% water change occurred whenever ammonia exceeded 1 mg/L (i.e., nearly daily) so that ammonia levels would not exceed 2.8 - 4.2 mg/L

Acclimatisation: 9 d

4. Test system:

Study type: Laboratory (static-renewal)

Guideline:
1. American Society for Testing and Materials (2003): Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, Philadelphia, Pennsylvania
2. U.S. Environmental Protection Agency (1996): Ecological Effects Test Guidelines: Special Considerations for Conducting Aquatic Laboratory Studies, OPPTS 850.1000. Office of Prevention, Pesticides and Toxic Substances, Washington, DC.

GLP: Not stated

Guideline deviations[23]:

1 a) In the acclimation phase feeding occurred ad libitum, it is not clear how often feeding is conducted, in the guideline it is recommended, that feeding has to occur at least once a day.

b) Silicone was used for the glass divider to separate the test chamber, it was recommended not to use silicone

c) Water quality parameters like hardness, alkalinity, conductivity is not presented, chemical oxygen demand (COD) is also missing but was only recommended to be desirable

d) No range finding test conducted, the range of WM concentrations was based on previously determined amphibian LC_{50} values for glyphosate herbicides[24,25])

e) Test species *Spea multiplicata* and *S. bombifrons* instead of the recommended organisms *Rana* sp. or *Bufo* sp.

f) Because only a single LC_{50} value was determined for each species-land use combination, 48- and 216-h LC_{50} values were compared by examining 84% confidence intervals for overlap (Jones et al., 2009). The same criteria was used to compare LC_{50} values between species and land uses.

g) Additional LC_1 is calculated, but no EC_{50} and IC_{50} is

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[23] Compared with Guideline 2002: American Society for Testing and Materials (2002): Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, Philadelphia, Pennsylvania

[24] Mann, R.M., Bidwell, J.R. (1999): The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Archives of Environmental Contamination and Toxicology, 36: 193 -199.

[25] Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N. (2004): Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry, 23: 1928 -1938.

	calculated
Duration of study:	Acute: starting time: 29th May (cropland larvae) 31st May (grassland larvae) Duration: 48 h Chronic: starting time: 29th and 31st May Duration: 30 d
Test conditions:	Test organisms were randomly distributed, test chamber of 37.9 L divided into two parts of 18.95 L, An 80% water change occurred every four days and herbicides were reapplied, moribund larvae were removed additional for acute testing: after 48 h survived animals were transferred into 5L water containers to check delayed death
Application:	Solution in each compartment was gently mixed with a clean glass rod
Application devices:	-
Water volume:	Acute: 18.95 L Chronic: 15 L
Treatments:	Acute: 8 Chronic: 2
Replicates per concentration:	Acute: 4 Chronic: 6
Individuals per replicate:	9, loading of organisms exceed not 0.5 g/L
Feeding during experiments:	Acute: no feeding the first 48 h Chronic: feeding with rabbit chow ad libitum
Parameters measured:	survival, mortality, LC_1, LC_{50} weight
Test concentrations:	Acute: 0.75, 1.5, 2.25, 3, 4.5, 6, 7.5, 10 mg glyphosate/L Chronic: 2.8 mg glyphosate ae[26]/L or 2.0 mg glyphosate ae/L.
Application / device:	-
Verification of dispersion:	Test solutions was determined by gas chromatography of the TMOA-derivatized products following Tseng et al. (2004)[27]
Validity criteria:	see guideline

5. Environmental conditions:

Test medium:	aerated aged tap water
Temperature:	mean: 21.96 ± 0.04°C (19.00 ± 0.04 – 22.65 ± 0.11 °C)
Photoperiod:	see "Holding conditions prior to test"
pH:	mean: 8.37 ± 0.02 (8.43 ± 0.04 – 8.75 ± 0.01)
Oxygen concentration/ saturation:	mean: 8.06 ± 0.08 (7.18 ± 0.06 – 8.35 ± 0.03 mg/L) (= 87 – 90 %)
Organic matter (C_{org}):	Not stated
Ammonium:	mean: 2.1 ± 0.1 mg/L (0.1 ± 0.0 – 0.3 ± 0.1 mg/L)
Conductivity:	Not stated

[26] acid equivalents
[27] Tseng, S.H., Lo, Y.W., Chang, P.C., Chou, S.S., Chang, H.M. (2004): Simultaneous quantification of glyphosate, glufosinate, and their major metabolites in rice and soybean sprouts by gas chromatography with pulsed flame photometric detector. Journal of Agricultural and Food Chemistry:,52: 4057- 4063.

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Hardness: Not stated
Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:
- Test procedure is comparable to guideline study (deviations are existing but acceptable and reference is made to literature)
- Sound statistical and experimental design.
- Environmental conditions are comparable to guideline study.
- Analytical determination included with reliable measurements of actual concentrations.
- Sound statistical and experimental design in both acute toxicity experiments and in the chronic toxicity experiment.

2. Relevance of study: **Relevant with restrictions**

Comment: This study is based on US national guidelines and matches most scientific principles. A well-documented report and sound statistical analysis make the study results relevant for general conclusions on the acute effect of this product on amphibians. Results comparable to another published study with this formulation tested against tadpoles (Fuentes et al. 2011)

3. Klimisch code: **Klimisch rating of 2**

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Author(s)	Year	Study title
Edginton, A.N., Sheridan, P.M., Stephenson, G.R., Thompson, D.G., Boermans, H.J.	2004	Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environmental Toxicology and Chemistry Volume: 23 Isssue: 4 Pages: 815-822

Abstract[28]

Vision®, a glyphosate-based herbicide containing a 15% (weight:weight) polyethoxylated tallow amine surfactant blend, and the concurrent factor of pH were tested to determine their interactive effects on early life-stage anurans. Ninety-six-hour laboratory static renewal studies, using the embryonic and larval life stages (Gosner 25) of *Rana clamitans*, *R. pipiens*, *Bufo americanus*, and *Xenopus laevis*, were performed under a central composite rotatable design. Mortality and the prevalence of malformations were modeled using generalized linear models with a profile deviance approach for obtaining confidence intervals. There was a significant ($p < 0.05$) interaction of pH with Vision concentration in all eight models, such that the toxicity of Vision was amplified by elevated pH. The surfactant is the major toxic component of Vision and is hypothesized, in this study, to be the source of the pH interaction. Larvae of *B. americanus* and *R. clamitans* were 1.5 to 3.8 times more sensitive than their corresponding embryos, whereas *X. laevis* and *R. pipiens* larvae were 6.8 to 8.9 times more sensitive. At pH values above 7.5, the Vision concentrations expected to kill 50% of the test larvae in 96-h (96-h lethal concentration [LC_{50}]) were predicted to be below the expected environmental concentration (EEC) as calculated by Canadian regulatory authorities. The EEC value represents a worst-case scenario for aerial Vision application and is calculated assuming an application of the maximum label rate (2.1 kg acid equivalents [a.e.]/ha) into a pond 15 cm in depth. The EEC of 1.4 mg a.e./L (4.5 mg/L Vision) was not exceeded by 96-h LC_{50} values for the embryo test. The larvae of the four species were comparable in sensitivity. Field studies should be completed using the more sensitive larval life stage to test for Vision toxicity at actual environmental concentrations.

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MATERIALS AND METHODS

1. Test material:

Test item(s):	Vision® (containing 15% MON 0818)
Active substance(s):	Glyphosate isopropylamine salt
Surfactant:	Polyethoxylated tallowamine (POEA)
Description:	For treatment of post-harvest areas in forestry to suppress the growth of undesirable competing vegetation; most commonly used in high-yield spruce/fir plantations.
Source of test substance:	Monsanto Company, Winnipeg, MB, Canada
Lot/Batch #:	PIT8903-301F
Purity:	356 g acid equivalent/L (glyphosate isopropylamine salt)
Stock solution:	Not stated
2. Vehicle and/or positive control:	none

3. Test organism:

[28] Quoted from article

Species:	*Xenopus laevis* (Anura, Pipidae) *Rana pipiens* (Anura, Ranidae) *Rana clamitans* (Anura, Ranidae) *Bufo americanus* (Anura, Bufonidae)
Age of test organisms at study initiation:	Embryo test: life stages at Gosner stage 8-10 Larval test: larvae at Gosner stage 25
Source:	*Xenopus laevis* from Hagen Aqualab, University of Guelph (Guelph, ON, Canada) *Rana pipiens, Rana clamitans,* and *Bufo americanus* were field collected.
Holding conditions prior to test:	*Xenopus laevis* adults were housed under flow-through conditions using filtered, irradiated well water at 18°C under 12:12 h light:dark cycle. Biweekly feeding rotation of beef liver and Frog Brittle®. *Rana pipiens, Rana clamitans,* and *Bufo americanus* embryos were held in 22°C culture water at a pH of 7.0 to 7.4 prior to testing.
Acclimatisation:	*Xenopus laevis* mating was stimulated by injection of 600 IU and 800 IU of human chorionic gonadotrophin. Amplexus, egg laying and fertilization occurred within 12 h in a 22°C, darkened room. The gelatinous coating of all embryos was removed using a 2% (w:v) cysteine solution in culture water and pH adjusted to 8.1.

4. Test system:

Study type:	Static renewal (24 h and 48 h intervals in embryo and larval test, respectively)
Guideline:	The American Society For Testing and Materials guideline: • Embryo test as well as the water used throughout the study conformed to the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) • Larval test according to document for the testing of larval amphibians.
GLP:	No
Guideline deviations:	In the embryo test, the FETAX was further adopted to the 3 other species.
Duration of study:	Embryo test: terminated when 90% of control reached G 25 which was by 96 hours for *X. laevis* and *B. americanus*, by 5 and 7 days for *R. pipiens* and *R. clamitans*, respectively. Larval test: 4 days.
Test conditions:	Embryo test: units consisted of 60 x 15 mm petri dish of 10 mL treatment solution at 23 ± 2°C. Assessments every 24 hours. Larval test: units were 2-L aquaria with 1 L treatment solution at 22 ± 1°C provided aeration. Assessments every 24 hours.
Treatments:	9 combinations of 5 pH levels (4.5, 5.0, 6.5, 8.0 and 8.5) and 5 test concentrations based on a midpoint for test concentrations from preliminary studies at pH 5.5. and 7.5. Untreated controls for each test (embryo and larval).
Test concentrations:	0.1 to 20 mg a.e./L
Replicates per treatment:	2 for each treatment combination but 10 for the midpoint treatment combination (i.e. medium test concentration at pH

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	6.5).
	2 – 4 for control (depending on availability of animals).
Organisms per replicate:	Embryo test: 20
	Larval test: 10
Feeding during experiments:	No feeding in embryo test and ad libitum in larval test (food mixture of Tetramin® and Spirulina® flakes).
Parameters measured:	Embryo test: mortality and hatching every 24 h, prevalence and malformations according to *Atlas of Abnormalities*[29] and total length at the end of test.
	Larval test: mortality and presence of gross malformations every 24 h, at the end, the length and weight of each surviving larvae.
Analytical determination of test concentrations:	Glyphosate and amimomethylphosphonic acid (primary degradation product) verified by gas chromatography.
Validity criteria:	Control mortality < 10%

5. Environmental conditions:

Test medium:	Water according to FETAX with adjusted pH using 1 N NaOH or 1 N HCl.
Photoperiod during tests:	Not stated
pH:	s. treatments
Oxygen saturation	> 80%
Composition:	625 mg NaCl, 96 mg NaHCO3, 30 mg KCl, 15 mg CaCl2, 60 mg CaSO4.2H2O, and 75 mg MgSO4/L of deionized water
Alkalinity:	Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment

- Test procedure is comparable to guideline study.
- Sound statistical and experimental design.

2. Relevance of study: **Relevant with restrictions**

Comment:

This study is based on US national guidelines and matches all scientific principles. A well-documented report and sound statistical analysis make the study results relevant for general conclusions on the acute effect of Vision on amphibians.

Because this study was performed not with the lead formulation its relevance is questionable to the evaluation.

3. Klimisch code: **Klimisch rating of 2.**

[29] Bantle, J.A., Dumont, J.N, Finch, R.A., Linder, G., and Fort, D.J. (1991). Atlas of abnormalities: A guide to the performance of FETAX. Technical Report. Oklahoma State Publications Department, Stillwater, OK, USA.

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Author(s)	Year	Study title
Edginton, A.N., Sheridan, P.M., Stephenson, G.R., Thompson, D.G., Boermans, H.J.	2004	Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environmental Toxicology and Chemistry Volume: 23 Isssue: 4 Pages: 815-822

Abstract[30]

Vision®, a glyphosate-based herbicide containing a 15% (weight:weight) polyethoxylated tallow amine surfactant blend, and the concurrent factor of pH were tested to determine their interactive effects on early life-stage anurans. Ninety-six-hour laboratory static renewal studies, using the embryonic and larval life stages (Gosner 25) of *Rana clamitans*, *R. pipiens*, *Bufo americanus*, and *Xenopus laevis*, were performed under a central composite rotatable design. Mortality and the prevalence of malformations were modeled using generalized linear models with a profile deviance approach for obtaining confidence intervals. There was a significant ($p < 0.05$) interaction of pH with Vision concentration in all eight models, such that the toxicity of Vision was amplified by elevated pH. The surfactant is the major toxic component of Vision and is hypothesized, in this study, to be the source of the pH interaction. Larvae of *B. americanus* and *R. clamitans* were 1.5 to 3.8 times more sensitive than their corresponding embryos, whereas *X. laevis* and *R. pipiens* larvae were 6.8 to 8.9 times more sensitive. At pH values above 7.5, the Vision concentrations expected to kill 50% of the test larvae in 96-h (96-h lethal concentration [LC_{50}]) were predicted to be below the expected environmental concentration (EEC) as calculated by Canadian regulatory authorities. The EEC value represents a worst-case scenario for aerial Vision application and is calculated assuming an application of the maximum label rate (2.1 kg acid equivalents [a.e.]/ha) into a pond 15 cm in depth. The EEC of 1.4 mg a.e./L (4.5 mg/L Vision) was not exceeded by 96-h LC_{50} values for the embryo test. The larvae of the four species were comparable in sensitivity. Field studies should be completed using the more sensitive larval life stage to test for Vision toxicity at actual environmental concentrations.

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MATERIALS AND METHODS

1. Test material:

Test item(s):	Vision® (containing 15% MON 0818)
Active substance(s):	Glyphosate isopropylamine salt
Surfactant:	Polyethoxylated tallowamine (POEA)
Description:	For treatment of post-harvest areas in forestry to suppress the growth of undesirable competing vegetation; most commonly used in high-yield spruce/fir plantations.
Source of test substance:	Monsanto Company, Winnipeg, MB, Canada
Lot/Batch #:	PIT8903-301F
Purity:	356 g acid equivalent/L (glyphosate isopropylamine salt)
Stock solution:	Not stated
2. Vehicle and/or positive control:	none
3. Test organism:	
Species:	*Xenopus laevis* (Anura, Pipidae)

[30] Quoted from article

	Rana pipiens (Anura, Ranidae) *Rana clamitans* (Anura, Ranidae) *Bufo americanus* (Anura, Bufonidae)
Age of test organisms at study initiation:	Embryo test: life stages at Gosner stage 8-10 Larval test: larvae at Gosner stage 25
Source:	*Xenopus laevis* from Hagen Aqualab, University of Guelph (Guelph, ON, Canada) *Rana pipiens, Rana clamitans,* and *Bufo americanus* were field collected.
Holding conditions prior to test:	*Xenopus laevis* adults were housed under flow-through conditions using filtered, irradiated well water at 18°C under 12:12 h light:dark cycle. Biweekly feeding rotation of beef liver and Frog Brittle®. *Rana pipiens, Rana clamitans,* and *Bufo americanus* embryos were held in 22°C culture water at a pH of 7.0 to 7.4 prior to testing.
Acclimatisation:	*Xenopus laevis* mating was stimulated by injection of 600 IU and 800 IU of human chorionic gonadotrophin. Amplexus, egg laying and fertilization occurred within 12 h in a 22°C, darkened room. The gelatinous coating of all embryos was removed using a 2% (w:v) cysteine solution in culture water and pH adjusted to 8.1.

4. Test system:

Study type:	Static renewal (24 h and 48 h intervals in embryo and larval test, respectively)
Guideline:	The American Society For Testing and Materials guideline: • Embryo test as well as the water used throughout the study conformed to the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) • Larval test according to document for the testing of larval amphibians.
GLP:	No
Guideline deviations:	In the embryo test, the FETAX was further adopted to the 3 other species.
Duration of study:	Embryo test: terminated when 90% of control reached G 25 which was by 96 hours for *X. laevis* and *B. americanus*, by 5 and 7 days for *R. pipiens* and *R. clamitans*, respectively. Larval test: 4 days.
Test conditions:	Embryo test: units consisted of 60 x 15 mm petri dish of 10 mL treatment solution at 23 ± 2°C. Assessments every 24 hours. Larval test: units were 2-L aquaria with 1 L treatment solution at 22 ± 1°C provided aeration. Assessments every 24 hours.
Treatments:	9 combinations of 5 pH levels (4.5, 5.0, 6.5, 8.0 and 8.5) and 5 test concentrations based on a midpoint for test concentrations from preliminary studies at pH 5.5. and 7.5. Untreated controls for each test (embryo and larval).
Test concentrations:	0.1 to 20 mg a.e./L
Replicates per treatment:	2 for each treatment combination but 10 for the midpoint treatment combination (i.e. medium test concentration at pH 6.5).

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	2 – 4 for control (depending on availability of animals).
Organisms per replicate:	Embryo test: 20 Larval test: 10
Feeding during experiments:	No feeding in embryo test and ad libitum in larval test (food mixture of Tetramin® and Spirulina® flakes).
Parameters measured:	Embryo test: mortality and hatching every 24 h, prevalence and malformations according to *Atlas of Abnormalities*[31] and total length at the end of test. Larval test: mortality and presence of gross malformations every 24 h, at the end, the length and weight of each surviving larvae.
Analytical determination of test concentrations:	Glyphosate and aminomethylphosphonic acid (primary degradation product) verified by gas chromatography.
Validity criteria:	Control mortality < 10%

5. Environmental conditions:

Test medium:	Water according to FETAX with adjusted pH using 1 N NaOH or 1 N HCl.
Photoperiod during tests:	Not stated
pH:	s. treatments
Oxygen saturation	> 80%
Composition:	625 mg NaCl, 96 mg NaHCO3, 30 mg KCl, 15 mg CaCl2, 60 mg CaSO4.2H2O, and 75 mg MgSO4/L of deionized water
Alkalinity:	Not stated

KLIMISCH EVALUATION

1. Reliability of study:	**Reliable**
Comment	• Test procedure is comparable to guideline study. • Sound statistical and experimental design.
2. Relevance of study:	**Relevant with restrictions**
Comment:	This study is based on US national guidelines and matches all scientific principles. A well-documented report and sound statistical analysis make the study results relevant for general conclusions on the acute effect of Vision on amphibians. Because this study was performed not with the lead formulation its relevance is questionable to the evaluation.
3. Klimisch code:	**Klimisch rating of 2**

[31] Bantle, J.A., Dumont, J.N, Finch, R.A., Linder, G., and Fort, D.J. (1991). Atlas of abnormalities: A guide to the performance of FETAX. Technical Report. Oklahoma State Publications Department, Stillwater, OK, USA.

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Author(s)	Year	Study title
Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N.	2004	Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry Volume: 23 Isssue: 8 Pages: 1928-1938

Abstract[32]

Glyphosate-based herbicides are among the most widely used pesticides in the world. We compared the acute toxicity of the glyphosate end-use formulation Roundup Original to four North American amphibian species (*Rana clamitans*, *R. pipiens*, *R. sylvatica*, and *Bufo americanus*) and the toxicity of glyphosate technical, the polyethoxylated tallowamine surfactant (POEA) commonly used in glyphosate-based herbicides, and five newer glyphosate formulations to R. clamitans. For R. clamitans, acute toxicity values in order of decreasing toxicity were POEA > Roundup Original > Roundup Transorb® > Glyfos AU®; no significant acute toxicity was observed with glyphosate technical material or the glyphosate formulations Roundup Biactive®, Touchdown®, or Glyfos BIO®. Comparisons between the four amphibian species showed that the toxicity of Roundup Original varied with species and developmental stage. *Rana pipiens* tadpoles chronically exposed to environmentally relevant concentrations of POEA or glyphosate formulations containing POEA showed decreased snout–vent length at metamorphosis and increased time to metamorphosis, tail damage, and gonadal abnormalities. These effects may be caused, in some part, by disruption of hormone signaling, because thyroid hormone receptor β mRNA transcript levels were elevated by exposure to formulations containing glyphosate and POEA. Taken together, the data suggest that surfactant composition must be considered in the evaluation of toxicity of glyphosate-based herbicides.

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MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup Original, RoundupTransorb®, Roundup Biactive (all from Monsanto), Touchdown (Syngenta), and Glyfos AU® and Glyfos BIO® (both from Cheminova, Wayne, NJ, USA)
Active substance(s):	360 g/L glyphosate isopropylamine salt in formulations and 570g/L in technical-grade glyphosate
Surfactant:	POEA in Roundup Original Blend with POEA in Roundup Transorb® Blend with 3-7% tallow alkylamine ethoxylate in Glyfos AU unspecified in Roundup Biactive, Glyfos BIO and Touchdown.
Description:	Several glyphosate-based herbicides with different chemistry and surfactant mixture are on the market. Toxicity to aquatic organisms and endocrine disrupting effects are supposed to depend on the recipe of formulations.
Source of test substance:	Glyphosate techn. (MON 0139), Polyethoxylated tallowamine (POEA as MON 0818), Roundup Biactive (MON 77920): Monsanto, St. Louis, MI, USA. Roundup Original (MON 78078), Roundup Transorb (MON): from local retailer, Canada.

[32] Quoted from article

	Glyfos AU®, Glyfos BIO® (CHA 4521): Cheminova, Wayne, NJ, USA. Touchdown® 480 (YF10251): Syngenta, Wilmington, DE, USA
Lot/Batch #:	Not stated
Purity:	360 g acid equivalent/L in formulations and 570 g acid equivalent/L in technical-grade glyphosate (i.e. formulation acid equivalents (FAE) corresponds to glyphosate isopropylamine salt)
Stock solution:	Prepared with deionized water right before exposure
2. Vehicle and/or positive control:	none
3. Test organism:	
Species:	*Rana clamitans* (Anura, Ranidae); acute *Rana pipiens* (Anura, Ranidae); acute and chronic *Rana sylvatica* (Anura, Ranidae); acute *Bufo americanus* (Anura, Bufonidae); acute.
Age of test organisms at study initiation:	Acute toxicity: at Gosner stage 20 and 25 Chronic toxicity: at Gosner stage 25
Source:	Field-collected from ponds along the Otonabee River (Canada) watershed within 5 km of Trent University (44°21'N, 78°17'W)
Holding conditions prior to test:	Eggs were collected within 24 h of being laid and kept under laboratory standard conditions. Tadpoles reared in glass aquaria with aerated and sand-filtered water from Otonabee River on a 12:12-h light cycle. Feeding tadpoles were provided with cooked lettuce and spinach ad libitum.
Acclimatisation:	none
4. Test system:	
Study type:	Static (acute toxicity) and static renewal (chronic toxicity)
Guideline:	Animal treatment according to guidelines of Trent University Animal Care Committee. Used water conformed to American Society for Testing and Materials[33].
GLP:	No
Duration of study:	Acute toxicity: 96 hours Chronic toxicity: 166 days including 42 days exposure.
Test conditions:	Acute toxicity: tested in 1-L glass beakers filled with filtered water with 24-h and 96-h assessments. A formulation comparison was done on *R. clamitans* and a species/stage comparison was done among all four species at two different life stages (Gosner 20 and 25). Chronic toxicity: tadpoles of *R. pipiens* were maintained at a density of 350 mL water per tadpole in aquaria. Exposure period of 42 days, followed by rearing in clean water. Experiment was terminated when at least 80% of surviving control tadpoles reach metamorphic climax at G 42.

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[33]American Society for Testing and Materials (2000): Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E729-96. In *Annual Book of Standards*. Vol 11.05, Section 11. Philadelphia, PA, pp. 220-240.

Treatments: Acute toxicity: 3 concentrations and 2 life stages in 1994 experiments for species and life stage comparison,
≥ 4 test concentrations of 6 formulations and 2 technical compounds (glyphosate and POEA) in 2001 experiment for formulation comparison,
and untreated control.

Chronic toxicity: 2 formulations (Roundup Original® and Roundup Transorb®) and 2 technical compounds (glyphosate and POEA) each at 2 test concentrations and an untreated control

Test concentrations: Acute toxicity: in formulation comparison, concentrations depend on formulation with a max. of 18 mg FAE/L; and in species/stage comparison 4, 6 and 8 mg FAE/L (equivalent to 12.9, 19.3 and 25.8 mg Roundup Original/L).

Chronic toxicity: 0.6 and 1.8 mg FAE/L

Replicates per treatment: 3 in acute toxicity

Organisms per replicate: 10 in 1994 experiments (acute toxicity for species/stage comparison)
20 in 2000/2001 experiments (acute toxicity for formulation comparison and chronic toxicity)

Feeding during experiments: Feeding stages were fed with cooked spinach (in acute formulation comparison) or cooked lettuce (

Parameters measured: Acute toxicity: 24-h and 96-h mortality. LC50 by trimmed Spearman-Kärber.

Chronic toxicity: Developmental rate until G42, tadpole length and tail damage, sex ratio, mRNA expression of transcription factors related to amphibian metamorphosis. Parametric (ANOVA) and non-parametric (chi-square, Fisher's exact test) null hypothesis tests for statistical comparisons.

Analytical determination of test concentrations: Results of study based on measured concentrations in 2000-2001 and on nominal concentrations in 1994.
Glyphosate and amimomethylphosphonic acid (primary degradation product) verified by high-performance liquid gas chromatography (HPLC-GC, 1994) and gas chromatography with nitrogen-phosphorus detection (GC, 2000-2001).

Validity criteria: Not stated

5. Environmental conditions:

Test medium: Filtered water from Otonabee River (published water chemistry data)

Temperature: 15 ± 1°C for *R. sylvatica*, *B. americanus*, and acute testing on *R. pipiens*.
20 ± 1°C for *R. clamitans* and chronic test on *R. pipiens*.

Photoperiod during tests: 12:12 h

pH: 7.8 – 8.3

Oxygen saturation At acceptable level according to guideline.

Composition: 11.6 mg/L dissolved organic carbon, 2.99 mg/L Na^+, 38 mg/L Ca^+, 3.5 mg/L Cl^-

Hardness: Not stated

Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment

- Environmental conditions are comparable to guideline study.
- Analytical determination included with reliable measurements of actual concentrations.
- Sound statistical and experimental design in both acute toxicity experiments and in the chronic toxicity experiment.
- High mortality of 38% was found in control for chronic toxicity testing decreasing the reliability of the results due to high natural variations.
- An extensive review of the histopathological findings and interpretation has been included in an additional review following the Klimisch rating below.

2. Relevance of study: **Relevant for acute toxicity experiments only and not chronic toxicity experiments**

Comment: The lead formulation in the re-evaluation was not tested, MON 52276.

Acute toxicity experiments can be considered to provide relevant results to characterize the acute toxicity of the test materials. However, significant comments regarding the relevance and validity of the histopathology findings from the chronic exposures are provided on the pages following the Klimisch code ratings.

3. Klimisch code: **Klimisch rating of 2 for acute data only. Klimisch rating of 3 for the chronic exposure data and not adequate for risk assessment. See extensive reviews provided below.**

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Expanded comments for Howe et al.

The goal of Howe *et al*. was to identify potential toxic effects of the herbicide glyphosate, the polyethoxylated surfactant (POEA), and selected formulations to several species of frogs that were given acute or chronic waterborne exposures. During acute exposures, mortalities were recorded to establish a 96-hour LC_{50} for one or more of the formulations in larval *Rana clamitans*, *R. pipiens*, *R. sylvatica*, and *Bufo americanus*. In contrast, chronic exposures (42-day exposure period) were conducted using only *R. pipiens*, and endpoints included: number of days to metamorphic climax; morphometric measurements (e.g., snout-vent length, total length, body length, tail height); degree of tail damage; gene expression (thyroid hormone-specific nuclear transcription factors); sex ratio; and gonadal histology).

In all but one of the treatment groups that included POEA in the formulation (each group consisting of 12-16 frogs), a low percentage of frogs (2-3 frogs per group) had reported patterns of gonadal development that were interpreted as intersex. There were no diagnoses of intersex in negative controls or in frogs treated with glyphosate technical. No changes were observed in sex ratio, either as compared to untreated controls or to historical data. The authors concluded that the intersex condition was induced by POEA-containing formulations.

This review includes general comments by the JGTF and a synopsis of key comments on the histopathological evaluation that were provided by an expert third party reviewer, Dr. Jeffery Wolf of EPL Incorporated. Dr. Wolf's full review has been included in this document as Appendix 1. Dr. Wolf is the leading expert in evaluating gonadal histopathology of aquatic vertebrates with recent and extensive experience working with fish and amphibian samples. Dr. Wolf has been involved in the validations for the fish and amphibian endocrine screening assays that are part of the EPA's Endocrine Disruption Screening Program.

<u>High ammonia levels and control mortality during chronic exposures is indicative of a high level of stress in the system</u>

A key issue with the chronic exposure is high control mortality, which was reported to be 38% by the end of the measurement period. Additionally, ammonia levels in the exposure tanks reached levels as high as 2.4 mg/L, which are considered to be unacceptably high in studies with aquatic vertebrates. Ammonia levels >1 mg/L combined with this high rate of control mortality question the validity of this study.

<u>Unrealistically high exposure levels were chosen for the chronic studies</u>

There are several important factors that must be considered when evaluating results from laboratory studies for their relevance to actual herbicide use and impact on non-target organisms, particularly when evaluating endocrine endpoints. Chronic exposure concentrations for the formulations were nominally 0.6 mg glyphosate acid equivalents/L and 1.8 mg glyphosate acid equivalents/L with weekly renewals over 6 weeks of continuous exposure. Chronic exposure concentrations for POEA in the Roundup Original® herbicide formulation were nominally 0.2 mg POEA/L and 0.6 mg POEA/L with weekly renewals for 6 weeks.[34] The higher test concentration chosen by Howe *et al*. corresponds to the highest measured glyphosate acid concentration in pond water reported by Giesy *et al.* (2000). This was an "instantaneous" concentration, measured soon after a direct over-spray application. In a real-world situation, dissipation of POEA from the water column would rapidly occur significantly limiting the duration of exposure at this level. Wang *et al*. (2005) demonstrated that DT_{50} values for POEA from the water column were less than one day, and dissipation correlates with decreased toxicity to an aquatic species.

It is also important to point out that these chronic exposure concentrations were a relatively large percentage of the reported 96 hour LC_{50} value for this species. The 96 hour LC_{50} value for Roundup

[34] The concentration of POEA in the treatment solutions was calculated for Roundup Original herbicide, for which the percentage of MON 0818 in the formulation is stated as approximately 15% (Howe *et al*. 2004, Table 1). MON 0818, however, is a surfactant blend containing 69-73% POEA (Howe *et al*. 2004, Table 1). Using the median percentage of 71%, the POEA concentration in the treatment solutions is 10.7% of the composition of Roundup Original herbicide. Since the glyphosate acid equivalent composition of the formulation was assumed to be 31%, the POEA concentration can be calculated as 34.5% of the glyphosate acid equivalent concentration (i.e. 0.2 and 0.6 mg POEA/L for the Roundup Original herbicide formulation).

Original herbicide with Gosner stage 25 tadpoles was reported to be 2.9 mg glyphosate acid equivalents/L, which would be equivalent to 1.0 mg POEA/L in the formulation. The chronic exposure concentrations were 0.6 and 1.8 mg glyphosate acid equivalents/L. Therefore, the higher exposure concentration was nearly two-thirds of the 96 hour LC50 value, and the lower exposure concentration was approximately one-fifth of the 96 hour LC50 value for this species.

There is no clear evidence of intersex in the photomicrographic figures

Because spermatogonia are not readily distinguishable from oogonia in histologic sections, identification of the testicular phenotype in immature gonadal tissue is primarily dependent on the recognition of a pattern in which primitive seminiferous tubules are surrounded by sheaths of medullary somatic cells. According to the text, Figures 2c and 2d are intended to represent two different manifestations of intersex. Although oocytes are clearly present in these two gonads, the remaining gonadal tissue is not overtly testicular in either example. Thus, rather than intersex, Figures 2c and 2d appear to depict ovaries that are merely less well-developed than the control ovary illustrated in Figure 2a.

Admittedly, because of low image resolution and contrast artifacts related to the printing process, published photomicrographic figures may not adequately portray changes viewed under the microscope. Therefore, diagnostic confirmation should be based on a re-evaluation of the histologic sections on glass slides.

Descriptions of normal gonad microanatomy and intersex are ambiguous or inaccurate

According to the text, "Normal testes of state 42 male metamorphs…were generally immature, containing spermatozoa surrounded by follicle cells with undefined seminiferous tubules," with Witschi (1929) being cited as the reference for that description. However, the term "follicle cells" is not typically used to describe cell types in the testes of any animal species and Witschi (1929) does not support that usage.

Spermatozoa were mentioned three times in association with metamorph testes, and this included the legends for Figures 2b and 2d. Spermatozoa are not apparent in either of those two photomicrographic figures, nor are intermediate developmental phases (i.e., spermatocytes and spermatids) present to any degree. Due to the level of gonad immaturity in frog metamorphs, it would be highly unlikely for spermatozoa to be found in either normal or abnormal testes of frogs at this developmental stage.

The stated descriptions of intersex are imprecise and thus potentially over-permissive. Mackenzie *et al.* (2003) (which included two of the authors from Howe *et al.*) provided the following characterization of intersex: "Intersex individuals were defined as frogs containing both ovarian and testicular gonadal tissue and germ cells." It is unclear why Howe *et al.* eschewed this straightforward definition of intersex in favor of the following criteria: "Individuals were categorized as intersex when we observed maturing primary oocytes…sometimes containing cortical alveoli, surrounded by varying degrees of somatic and/or medullary tissue." The phrase "somatic and/or medullary tissue" was not further defined, and the morphologic appearance of such tissue was not described. Although the testis is derived from the primordial gonadal medulla, the word "somatic", which refers to non-germinative tissues, is not inherently gender specific; therefore, when used independently, that word does not provide a meaningful contribution to the diagnosis of intersex.

One potentially convincing presentation of intersex described in this paper was the finding of "gonads with ovarian tissue at the posterior end and testicular tissue at the anterior end"; however, a representative photomicrograph of that particular manifestation was not provided, and, thus, the accuracy of that diagnosis can not be confirmed. Two other morphologic patterns presumed to represent intersex included "an abnormal lining of an ovarian cavity" and "an enlarged germinal epithelium with a proliferation of oogonia and atretic oocytes." Although the authors indicated that the latter pattern was observed previously by Yü *et al.* (1972), there is no evidence that a similar description exists within that paper.

The prevalence of intersex was not significantly greater in treated animals when compared to controls

Statistical analyses were performed for virtually all assessed parameters other than the prevalence of intersex, and no reason was given for excluding that particular result. Had the data been tested using a Fisher's Exact test, the prevalence of findings diagnosed as intersex would not have been significantly

different (p<0.05) from controls for any of the treatment groups. By itself, the lack of significant difference does not necessarily indicate an absence of treatment effect, especially if there is evidence to suggest that intersex never occurs spontaneously in *R. pipiens* experimental controls. However, that is patently not the case, as indicated in other laboratory studies in which leopard frog controls were examined at various stages of somatic (and reproductive tract) development (Mackenzie *et al*., 2003; Hogan *et al*., 2008; Jofré and Karasov, 2008; Orton *et al*., 2006).

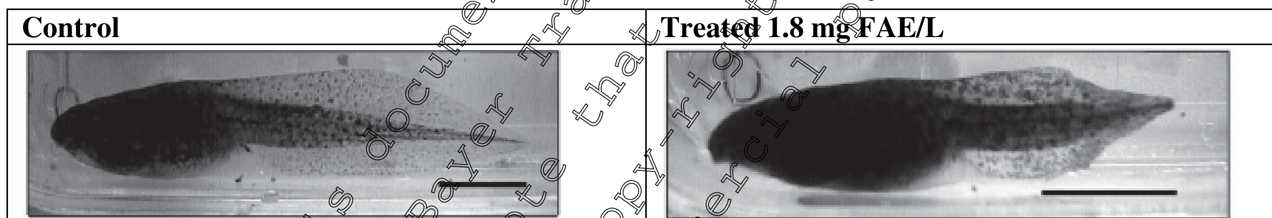
<u>Alternative causes for gonadal changes were not considered</u>

Developmental delays coupled with overt signs of toxicity likely indicate a non-specific toxic effect (OECD Test Guideline 231, OECD 2009). Therefore, evaluation of non-thyroidal toxicity in chronic studies is essential to reduce the probability of false positives which can result from a non-specific effect.

The chronic exposure by Howe *et al*. was initiated with Gosner stage 25 tadpoles. Consequently, a significant portion of the exposure occurred during pre-metamorphosis, which could have affected thyroid gland development through non-thyroidal mechanisms and growth and development. The observed effects on growth appear to be largely related to an apparent effect of the membrane disrupting properties of the surfactant and not directly through an endocrine mediated mechanism. The mode of action of surfactants to aquatic organisms is generally accepted to be nonspecific, with exposure resulting in disruption of biological membrane integrity (Roberts, 1991; Roberts and Marshall, 1995).

A review of Figure 4 in Howe *et al*. demonstrates the severe tail damage caused by these high level chronic exposures; this damage is consistent with the membrane disrupting action of surfactants on tissues. Therefore, the reported reductions in growth strongly suggest a non-specific toxic effect. Growth should never be solely relied upon to determine thyroid toxicity. Rather, growth, in conjunction with developmental stage and thyroid histopathology, should be used to determine thyroid activity. Other endpoints should also be considered in determining overt toxicity including severe tissue damage as shown in Figure 4 of this study.

Control	Treated 1.8 mg FAE/L



In the article introduction, the authors pointed out that "several nonionic surfactants (i.e., nonyl-and octylphenols) may act as endocrine-disrupting compounds (EDCs) and have shown estrogenicity in EDC assays." However, the authors neglected to mention that POEA and alkylphenols do not share similar structural motifs, which are estrogenic, or whether POEA had ever been demonstrated to have estrogenic effects, thereby leaving the reader with an impression of "guilt by association." Typically, compounds that have estrogenic activity contain at least one cycle (e.g., benzene ring), and POEA does not have this motif rather it has a long alkyl chain, which is not indicative of estrogenicity.

In the discussion section, the authors postulated that gonadal abnormalities may have been caused by POEA-induced alterations of the thyroid axis, which in turn led to enhanced estrogenic effects on the gonads. Paradoxically, they also noted that "past studies have shown that changes in thyroid hormone levels do not affect gonadal development directly." Although they ultimately conceded that "the exact mechanism of the abnormal sexual development induced by Roundup Original, Roundup Transorb, and POEA is unclear…," they did not entertain theories other than the speculated POEA-thyroid-estrogen connection. However, they stated that it "is interesting to note that Roundup can inhibit steroidogenesis by interfering with the transport of cholesterol across the mitochondrial membrane (Walsh *et al*., 2000)." A follow up study demonstrated that the results reported by Walsh *et al*. resulted from the non-specific action of a variety of surfactants at supra-physiological concentrations on cellular function in an *in vitro* test system (Levine *et al*., 2007).

Results of the acute and chronic experiments in Howe *et al*. indicated that the tested concentrations of POEA were capable of causing systemic and dermal toxicity, respectively. In the chronic trial, it is not

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unreasonable to assume that prolonged illness associated with the necrotizing lesions in the tail (and perhaps additional tissues that were not investigated) was physiologically stressful for the tadpoles. It is well established that stress, whether induced by adverse environmental factors (e.g., high stocking density, osmotic compromise, dietary modification, nitrate toxicity) or mimicked via glucocorticoid administration, is capable of impeding anuran larval growth and development (Glennemeier and Denver, 2002; Gomez-Mestre *et al*., 2004; Ledón-Rettig *et al*, 2003; Edwards *et al*., 2006; Yü *et al*., 1972). Accordingly, it is not surprising that somatic growth and metamorphosis were delayed by POEA treatment in the chronic experiment, and it should be recognized that these delays need not have required a direct effect of POEA on the thyroid axis.

Although the authors briefly mentioned stress as a possible cause (rather than consequence) of contaminant-induced tail damage, they did not address the potential effect of stress on time-to-metamorphosis and snout-vent growth. Furthermore, it is unlikely that stress effects would have been limited to somatic tissues. Possible consequences of stress in the developing reproductive tract could have included maturation delay and oocyte atresia, the latter of which was observed in an unreported percentage of POEA-treated frogs. In at least some treated frogs, the degree of gonadal maturation delay might have exceeded metamorphic delay, and thus the gonads of these animals would have appeared relatively immature compared to other Gosner Stage 42 froglets. In the developing ovary, maturation delay would manifest as an increased proportion of oogonia relative to perinucleolar oocytes, when compared to the ovaries of control females (similar to the appearance of gonads in Figures 2c and 2d). Potentially, when viewed alongside the ovaries of control frogs, such comparatively underdeveloped ovaries could have been mischaracterized as intersex.

Assuming that the diagnoses of intersex in Howe *et al*. were in fact accurate, it is still possible that the existence of intersex in these frogs was caused by a stress-induced delay in gonadal maturation. According to previous investigators, the developing gonads of pre-metamorphic *R. pipiens* (and certain other anurans) may proceed naturally through an indifferent phase (transitory juvenile hermaphroditism) in which male and female elements co-exist temporarily within the same gonad (Hogan *et al*., 2008; Jofré and Karasov, 2008; Eggert, 2004). Notably, delayed gonadal maturation was cited as a potential mechanism for PCB-induced intersex in leopard frogs (Jofré and Karasov, 2008).

<u>Certain details of the histologic examination were not reported</u>

In Howe *et al*., the Materials and Methods section does not contain certain procedural details that would help the reader to assess, to some degree, the quality of the microscopic examinations that were performed. Such details include the number of people who evaluated the histologic sections and their level(s) of expertise, the average number of sections that were microtomed and evaluated per frog, and whether the evaluator was aware of the treatment-group status of individual frogs during the examinations.

Regarding the number of sections per frog, the text states that "transverse-step sections (thickness, 7 µm) were cut through the entire area of the gonad…." There is no statement that indicates whether these were actually serial sections (i.e., every section in the microtomed tissue ribbon mounted and stained) or whether intervals of tissue between these sections were discarded. Assuming that they were in fact serial sections, and also assuming conservatively that the average gonad length in Gosner Stage 42 frogs is approximately 3 mm, the number of 7 µm transverse sections produced per frog would have been > 42,000. Multiplying that number by 114 (the number of frogs in the chronic toxicity segment of the study) produces a total of 4.8 million histologic sections. If that value does not approximate the number of sections that were actually examined, then the histologic assessment was less complete than might be initially construed from the text.

In many toxicologic bioassays, the treatment group status of individual animals is masked ("blinded") in order to prevent test operators from consciously or subconsciously introducing bias into the results. In histopathologic evaluations, blinding is generally not recommended during the initial slide evaluation, because it can allow subtle morphologic changes to go unnoticed, and thus produce false-negative results (Crissman *et al*., 2004). On the other hand, once all relevant changes have been identified, a second reading using masked slides should be performed to confirm each test article-related diagnosis, in order to guard against potential false-positive results. There is no information in Howe *et al*. to indicate whether

findings were either evaluated or re-evaluated in a blinded manner, and thus the potential for bias inclusion remains uncertain.

The prevalence and severity of oocyte atresia were not reported

Oocyte atresia is mentioned in Howe *et al*. as a feature of gonads that were diagnosed as intersex or were otherwise considered to be abnormal. However, it is important to recognize that atresia can occur as a normal physiologic process or a non-specific pathologic response, depending on the stimulus. Consequently, because atresia can occur independent of intersex or any other condition, this finding should be recorded and reported separately. In this study, it might have been useful to know whether the prevalence and severity of atresia were greater in POEA-treated tadpoles vs. controls.

At one point in the discussion section of Howe *et al*., the authors describe stress as a hormone disruption mechanism because the effects of stress are mediated through corticosteroid hormones. While this is technically correct, it is currently the case that substances that primarily influence the HPT (thyroid) or HPG (reproductive) axis are often regarded and regulated differently from compounds that affect the HPA (adrenal) axis secondarily via toxicologically-induced stress. Thus, it is imperative that data used to provide this type of mechanistic information be as accurate as possible.

Expression analysis of TRβ mRNA levels in tail tissue is not indicative of endocrine toxicity

No significant effect on TRβ mRNA levels in tail tissue was detected at Gosner stages 25 and 42 after chronic exposure to MON 0818. However, a small (< 2-fold) but statistically significant increase in TRβ mRNA levels in tail tissue was detected only in Gosner stage 25 tadpoles after chronic exposure to the higher concentration of Roundup Original. Considering that the exposure was performed at a concentration that resulted in extensive tail necrosis, an interpretation of changes in TRβ mRNA levels is confounded.

Conclusions from the review of Howe *et al.* (2004)

The results of Howe *et al*. need to be viewed with extreme caution because the chronic exposure levels used in their study (42 days) greatly exceed realistic chronic exposures in the environment. POEA rapidly dissipates in the aquatic environment resulting in chronic exposures at levels much lower than those used in Howe *et al*. Furthermore, based on an extensive review of this paper, the strength of the evidence for adverse effects through a specific endocrine mechanism particularly at environmentally realistic concentrations is scientifically weak. Therefore, the endpoints from this study are not considered to be acceptable for use in an ecological assessment.

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A Critical Review of Gonadal Histopathology Procedures, Results, and Interpretations in Howe *et al*. (2004)

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A Critical Review of Gonadal Histopathology Procedures, Results, and Interpretations in Howe et al. (2004)

Background

The goal of Howe et al. was to identify potential toxic effects of the herbicide glyphosate, the polyethoxylated surfactant (POEA), and various combinations of these two chemicals on several species of frogs that were exposed acutely or chronically via the aqueous route under laboratory conditions. During acute exposures, mortalities were recorded to establish an LC50 for one or more of the formulations in larval *Rana clamitans*, *R. pipiens*, *R. sylvatica*, and *Bufo americanus*. In contrast, chronic exposures (42-day exposure period) were conducted using *R. pipiens* only, and endpoints included: number of days to metamorphic climax; morphometric measurements (e.g., snout-vent length, total length, body length, tail height); degree of tail damage; gene expression (thyroid hormone-specific nuclear transcription factors); sex ratio; and gonadal histology.

In all but one of the treatment groups that included POEA in the formulation (each group consisting of 12-16 frogs), a low percentage of frogs (2-3 frogs per group) had patterns of gonadal development that were interpreted as intersex. There were no diagnoses of intersex in negative controls or in frogs treated with glyphosate technical. No changes were observed in sex ratio, either as compared to untreated controls or to historical data. The authors concluded that the intersex condition was induced by POEA-containing formulations.

The purpose of this critique is to evaluate the histopathologic descriptions and interpretations presented in Howe et al. The decision to create this written assessment was based on a preliminary review in which a number of key problems were identified. These are prioritized into issues of greater and lesser concern.

Issues of Greater Concern

1. There is no clear evidence of intersex in the photomicrographic figures.

Because spermatogonia are not readily distinguishable from oogonia in histologic sections, identification of the testicular phenotype in immature gonadal tissue is primarily dependent on the recognition of a pattern in which primitive seminiferous tubules are surrounded by sheaths of medullary somatic cells. According to the text, Figures 2c and 2d are intended to represent two different manifestations of intersex. Although oocytes are clearly present in these two gonads, the remaining gonadal tissue is not overtly testicular in either example. Thus rather than intersex, Figures 2c and 2d appear to depict ovaries that are merely less well-developed than the control ovary illustrated in Figure 2a.

Admittedly, because of low image resolution and contrast artifacts related to the printing process, published photomicrographic figures may not adequately portray changes viewed under the microscope. Therefore, diagnostic confirmation should be based on a re-evaluation of the histologic sections on glass slides.

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2. **Descriptions of normal gonad microanatomy and intersex are ambiguous or inaccurate.**

   a) According to the text, "Normal testes of state 42 male metamorphs…were generally immature, containing spermatozoa surrounded by follicle cells with undefined seminiferous tubules." and Witschi (1929), was cited as the reference for that description. However, the term "follicle cells" is not typically used to describe cell types in the testes of any animal species, and Witschi (1929) does not support that usage.

   b) Spermatozoa were mentioned three times in association with metamorph testes and this included the legends for Figures 2b and 2d. Spermatozoa are not apparent in either of those two photomicrographic figures, nor are intermediate developmental phases (i.e., spermatocytes and spermatids) present to any degree. Due to the level of gonad immaturity in frog metamorphs, it would be highly unlikely for spermatozoa to be found in either normal or abnormal testes of frogs at this developmental stage.

   c) The stated descriptions of intersex are imprecise and thus potentially over-permissive. Mackenzie et al. (2003) (which included two of the authors from Howe et al.) provided the following characterization of intersex: "Intersex individuals were defined as frogs containing both ovarian and testicular gonadal tissue and germ cells." It is unclear why Howe et al. eschewed this straightforward definition of intersex in favor of the following criteria: "Individuals were categorized as intersex when we observed maturing primary oocytes…sometimes containing cortical alveoli, surrounded by varying degrees of somatic and/or medullary tissue." The phrase "somatic and/or medullary tissue" was not further defined, and the morphologic appearance of such tissue was not described. Although the testis is derived from the primordial gonadal medulla, the word "somatic", which refers to non-germinative tissues, is not inherently gender specific; therefore, when used independently, that word does not provide a meaningful contribution to the diagnosis of intersex.

   One potentially convincing presentation of intersex described in this paper was the finding of "gonads with ovarian tissue at the posterior end and testicular tissue at the anterior end"; however, a representative photomicrograph of that particular manifestation was not provided, and thus the accuracy of that diagnosis could not be confirmed. Two other morphologic patterns presumed to represent intersex included "an abnormal lining of an ovarian cavity" and "an enlarged germinal epithelium with a proliferation of oogonia and atretic oocytes". Although the authors indicated that the latter pattern was observed previously by Yu et al. (1972), there is no evidence that a similar description exists within that paper.

3. **The prevalence of intersex was not significantly greater in treated animals when compared to controls.**

Statistical analyses were performed for virtually all assessed parameters other than the prevalence of intersex, and no reason was given for excluding that particular result. Had the data been tested using a Fisher's Exact test, the prevalence of findings diagnosed as intersex would not have been significantly different ($p<0.05$) from controls for any of the treatment groups. By itself, the lack of significant difference does not necessarily indicate an absence of treatment effect, especially if there is evidence to suggest that intersex never occurs spontaneously in *R. pipiens* experimental controls. However, that is patently not the case, as indicated in other laboratory studies in which leopard frog controls were examined at various stages of somatic (and reproductive tract) development (Mackenzie et al., 2003; Hogan et al., 2008; Jofré and Karasov, 2008; Orton et al., 2006).

4. Alternative causes for gonadal changes were not considered.

In the article introduction, the authors pointed out that "several nonionic surfactants (i.e., nonyl-and octylphenols) may act as endocrine-disrupting compounds (EDCs) and have shown estrogenicity in EDC assays." However, the authors neglected to mention whether POEA and alkylphenols share similar structural motifs, or whether POEA had ever been demonstrated to have estrogenic effects, thereby leaving the reader with an impression of "guilt by association".

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Results of the acute and chronic experiments in Howe et al. indicated that the tested concentrations of POEA were capable of causing systemic and dermal toxicity, respectively. In the chronic trial, it is not unreasonable to assume that prolonged illness associated with the necrotizing lesions in the tail (and perhaps additional tissues that were not investigated) was physiologically stressful for the tadpoles. It is well established that stress, whether induced by adverse environmental factors (e.g., high stocking density, osmotic compromise, dietary modification, nitrate toxicity) or mimicked via glucocorticoid administration, is capable of impeding anuran larval growth and development (Glennemeier and Denver, 2002; Gomez-Mestre et al., 2004; Ledón-Rettig et al, 2009; Edwards et al., 2006; Yu et al., 1972). Accordingly, it is not surprising that somatic growth and metamorphosis were delayed by POEA treatment in the chronic experiment, and it should be recognized that these delays need not have required a direct effect of POEA on the thyroid axis.

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Although the authors briefly mentioned stress as a possible cause (rather than consequence) of contaminant-induced tail damage, they did not address the potential effect of stress on time-to-metamorphosis and snout-vent growth. Furthermore, it is unlikely that stress effects would have been limited to somatic tissues. Possible consequences of stress in the developing reproductive tract could have included maturation delay and oocyte atresia, the latter of which was observed in an unreported percentage of POEA-treated frogs. In at least some treated frogs, the degree of gonadal maturation delay might have exceeded metamorphic delay, and thus the gonads of these animals would have appeared relatively immature compared to other Gosner Stage 42 froglets. In the developing ovary, maturation delay would manifest as an increased proportion of oogonia relative to perinucleolar oocytes, when compared to the ovaries of control females (similar to the appearance of gonads in Figures 2c and 2d). Potentially, when viewed alongside the ovaries of control frogs, such comparatively underdeveloped ovaries could have been mischaracterized as intersex.

Assuming that the diagnoses of intersex in Howe et al. were in fact accurate, it is still possible that the existence of intersex in these frogs was caused by a stress-induced delay in gonadal maturation. According to previous investigators, the developing gonads of pre-metamorphic *R. pipiens* (and certain other anurans) may proceed naturally through an indifferent phase (transitory juvenile hermaphroditism) in which male and female elements co-exist temporarily within the same gonad (Hogan et al., 2008; Jofré and Karasov, 2008; Eggert, 2004). Notably, delayed gonadal maturation was cited as a potential mechanism for PCB-induced intersex in leopard frogs (Jofré and Karasov, 2008).

Issues of Lesser Concern

1. Certain details of the histologic examination were not reported.

In Howe et al., the materials and methods section does not contain certain procedural details that would help the reader to assess, to some degree, the quality of the microscopic examinations that were performed. Such details include the number of people who evaluated the histologic sections and their level(s) of expertise, the average number of sections that were microtomed and evaluated per frog, and whether the evaluator was aware of the treatment-group status of individual frogs during the examinations.

Regarding the number of sections per frog, the text states that "transverse-step sections (thickness, 7 μm) were cut through the entire area of the gonad…" There is no statement that indicates whether these were actually serial sections (i.e., every section in the microtomed tissue ribbon mounted and stained), or whether intervals of tissue between these sections were discarded. Assuming that they were in fact serial sections, and also assuming conservatively that the average gonad length in Gosner Stage 42 frogs is approximately 3 mm, the number of 7 μm transverse sections produced per frog would have been > 42,000. Multiplying that number by 114 (the number of frogs in the chronic toxicity segment of the study) produces a total of 4.8 million histologic sections. If that

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value does not approximate the number of sections that were actually examined, then the histologic assessment was less complete than might be initially construed from the text.

In many toxicologic bioassays, the treatment group status of individual animals is masked ("blinded") in order to prevent test operators from consciously or subconsciously introducing bias into the results. In histopathologic evaluations, blinding is generally not recommended during the initial slide evaluation, because it can allow subtle morphologic changes to go unnoticed, and thus produce false-negative results (Crissman et al., 2004). On the other hand, once all relevant changes have been identified, a second reading using masked slides should be performed to confirm each test article-related diagnosis, in order to guard against potential false-positive results (Crissman et al., 2004). There is no information in Howe et al. to indicate whether findings were either evaluated or re-evaluated in a blinded manner, and thus the potential for bias inclusion remains uncertain.

2. The prevalence and severity of oocyte atresia were not reported.

Oocyte atresia is mentioned in Howe et al. as a feature of gonads that were diagnosed as intersex or were otherwise considered to be abnormal. However, it is important to recognize that atresia can occur as a normal physiologic process or a non-specific pathologic response, depending on the stimulus. Consequently, because atresia can occur independent of intersex or any other condition, this finding should be recorded and reported separately. In this study, it might have been useful to know whether the prevalence and severity of atresia were greater in POEA-treated tadpoles vs. controls.

Further Discussion and Recommendations

At one point in the discussion section of Howe et al., the authors describe stress as a hormone disruption mechanism because the effects of stress are mediated through corticosteroid hormones. While this is technically correct, it is currently the case that substances that primarily influence the HPT (thyroid) or HPG (reproductive) axis are often regarded and regulated differently from compounds that affect the HPA (adrenal) axis secondarily via toxicologically-induced stress. Thus, it is imperative that data used to provide this type of mechanistic information be as accurate as possible.

As stated previously, definitive conclusions regarding the accuracy of histopathologic diagnoses should not be based solely on published photomicrographs, which offer only a keyhole view of the histologic material, and frequently suffer from artifactual distortion. Instead, it is recommended that a relevant subset of the original glass slides from Howe et al. be reviewed in a formal manner using a mechanism known as pathology peer review.

Not to be confused with journal peer review, in which the correctness of the raw data cannot usually be verified, pathology peer review is a non-adversarial procedure used to ensure that the histopathologic results are of the highest quality in terms of diagnostic accuracy, terminology, and consistency. In keeping with currently accepted peer review practices (The Society of Toxicologic Pathologists, 1991), the following procedures are advocated for a review of histologic slides from the Howe et al. study:

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1. A selected subset of the slides should be examined by a highly trained toxicologic anatomic pathologist who has demonstrated expertise in the diagnosis of reproductive endocrine disruption lesions in the gonads of anurans and other aquatic animals.

2. Slides should be selected on the following basis:
   a. To allow the pathologist to appreciate the range of normal variability, all slides from all control animals should be examined.
   b. To confirm the validity of the intersex diagnosis, the pathologist should examine all slides from all animals that had an original determination of intersex.
   c. To determine if additional treatment-related morphologic changes might not have been appreciated, all slides from a predetermined fraction (e.g., 10%) of randomly selected POEA-treated animals should be examined.

3. The reviewing pathologist should have access to all pertinent data for the individual animals to be reviewed, including the original histology results, plus data representing growth and development endpoints.

4. Because pathology peer review is essentially a quality assurance device, and not a re-read of the entire study, the review should be performed in a non-blinded manner. To promote confidence in the results, the review should be conducted and documented in accordance with Good Laboratory Practice guidelines.

5. If diagnostic discrepancies are found to exist between the reviewing pathologist and the scientist who originally read the slides, these can be resolved potentially via mutual agreement. If agreement between these two parties cannot be reached regarding one or more key diagnoses, then the slides in question should be reviewed by a panel of experts (pathology working group) tasked with resolving such differences by majority vote.

8-5-10

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Author(s)	Year	Study title
Jayawardena, U.A., Rajakaruna, R.S., Nararatne, A.N., Amerasinghe, P.H.	2010	Toxicity of agrochemicals to Common hourglass tree frog (*Polypedates cruciger*) in acute and chronic exposure International Journal of Agriculture and Biology Volume: 12 Pages: 641-648 ISSN: 1560-8530 (print), 1814-9596 (online)

Abstract[35]

Direct effect of four common agricultural pesticides viz., chlorpyrifos, dimethoate, glyphosate and propanil, on the survival, growth and development of malformations in common hourglass tree frog, *Polypedates cruciger* (Anura: Ranidae) was studied under laboratory conditions in acute and chronic exposure. Acute exposure to high concentrations was carried out to determine the LC_{50}. The 48 h LC_{50} of the pesticides were within the Pesticide Area Network specified limits, except for propanil. The percentage survival of the tadpoles under chronic exposure to ecologically relevant doses was lower (glyphosate 75%, dimethoate 77.5%, chlorpyrifos 80% & propanil 85%) than the control group (95.5%) and was significantly affected by the concentrations. Exposed tadpoles took more time to metamorphose and were significantly smaller in size than the control tadpoles. They also developed malformations at high frequencies (glyphosate = 69%, dimethoate = 64%, chlorpyrifos = 60%, propanil = 45%). Malformations were mainly kyphosis (hunched back), scoliosis (curvature), skin ulcers and edema. However, severe limb malformations were not observed in the study. Chlorpyrifos had a profound effect even at very low concentrations (0.05 ppm). This study provides the first empirical evidence of a comparative study on the effect of pesticides on an endemic amphibian species in Sri Lanka and underscores the importance of investigation the level of agricultural pesticides in freshwater ecosystems and their effect on non-target organisms.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup® or Glyphosate®
Active substance(s):	
Surfactant:	Not stated
Description:	Roundup® or Glyphosate® is for use in coconut plantations at 1.44 kg/ha in Sri Lanka.
Source of test substance:	Pesticide Registrar's Office, Peradeniya, Sri Lanka
Lot/Batch #:	Not stated
Purity:	Not stated
Stock solution:	Not stated

2. Vehicle and/or positive control: Not stated; but tadpoles were exposed to three more commercially available pesticides.

3. Test organism:

Species:	*Polypedates cruciger* (Anura, Rhacophoridae)
Age of test organisms at study initiation:	5 days after hatching (ie. Gosner stage 25-26)

[35] Quoted from article

Source: Newly spawned foamy egg masses were field-collected from Peradeniya University Park, Sri Lanka

Holding conditions prior to test: Eggs were kept in glass with dechlorinated tap water, emergent tadpoles were fed with commercial fish feed three times/day.

Acclimatisation: Not stated

4. Test system:

Study type: Static (acute) and static renewal (chronic)

Guideline: None

GLP: No

Duration of study: 48 h (acute), and > 30 days (chronic; until metamorphosis)

Test conditions: Glyphosate was tested amongst other pesticides but in separate treatments. The product diluted in dechlorinated water was exposed directly to tadpoles in glass tanks (15 x 15 x 25 cm) containing 2 L test solution. In chronic study, water was renewed every week and tadpoles were fed with commercial fish feed.
In acute studies, mortality was assessed after 48 h. In the chronic study, malformations were recorded at 10 d and 30 d post-hatch (Gosner 27 and 31) and at metamorphosis.

Treatments: 5 concentrations and untreated water control (dechlorinated tap water) for acute exposure, 4 concentrations and an untreated control for chronic exposure.

Test concentrations: Acute: 9.50, 11.25, 15.00, 18.75 and 25.0 mg/.
Chronic: 0.25, 0.50, 0.75 and 1.00 mg/L

Replicates per concentration/control: Unclear; three of four clutches were selected and 20 individuals placed in each glass tank.

Organisms per replicate: 20 per tank

Feeding during experiments: 3 times/day with commercial fish feed.

Parameters measured: Acute toxicity: 48-h mortality. LC_{10}, LC_{50} and LC_{90} by probit analysis.
Chronic toxicity: Malformations of snout-vent length (SVL), time required until forelimb emergence of half of tadpoles (TE_{50}).

Analytical determination of test concentrations: None

Validity criteria: Not stated

5. Environmental conditions:

Test medium: Dechlorinated tap water

Temperature: 27-31°C

Photoperiod during tests: 12:12 h

pH: Not stated

Oxygen saturation Not stated

Composition: Not stated

Hardness: Not stated

Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment

- The glyphosate formulation used in testing is referred to by the authors both as "Roundup®" and "Glyphosate®." It is unclear if there was one or two different formulations tested and which may have been used for acute and chronic tests. The fact that the acute LC_{50} was 14.99 mg/L, but 25% mortality was observed in the chronic study at 1 mg/L, suggests that two different formulations may have been used because the slope of the dose response curve for glyphosate formulations is generally very steep.
- It is not indicated whether endpoints are reported in units of formulation, glyphosate salt or glyphosate acid, and there is no analytical confirmation of test concentrations reported.
- No water quality parameters are reported. It is indicated that the tadpoles were fed three times per day with commercial fish food, yet the medium was only changed once per week. In addition, glyphosate formulations can change the pH of aqueous solutions with the degree of change dependent on water hardness. There is no information on the pH of the medium or the concentration of toxic components such as ammonia.
- The experimental design for the acute study is never clearly described in the materials and methods section. For example, under the "Acute exposure to determine LC_{50} values" section the authors state that three of four clutches were selected and 20 individuals were placed in a glass tank, however it is not equivocally stated whether 3 replicates each with 20 tadpoles was used, this is merely implied.
- There is insufficient information provided in the paper regarding test conditions and acute and chronic exposure study designs to support the conclusions drawn.
- The statistical treatment and illustration of the results in Figure 1 for the time to 50% emergence (TE_{50}) is misleading and confusing. An F-test (e.g., one way ANOVA) was employed for comparison of treatment mean values; however, a post-hoc statistical analysis (e.g. means separation test) was not performed. Therefore, the authors could not distinguish between a treatment effect and a specific effect at a given concentration level. Moreover, visual comparison of the 0.5 ppm glyphosate treatment with the control TE_{50} (Figure 1) indicates virtually identical values, and, given the indicated data variation, it is unclear how a statistical comparison identified a significant difference, further questioning the statistical treatment of the data. It should also be noted that the glyphosate treatments are not identified sequentially by magnitude, but rather go from 0.5 to 0.25, then to 0.75 and 1 ppm in the figure legend adding further

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confusion to the interpretation of the results.

- The calculations to determine the percentage of malformation artificially inflate the number of malformations. The authors divided the number of malformed individuals by the number of surviving individuals in a treatment. The authors should have divided the number of malformed individuals by the starting number of individuals in a treatment to appropriately represent the number of malformations per treatment.
- The data outlined in Figure 3 pertaining to the incidence of malformation are quite particular given the variation surrounding the controls. Based on visual interpretation of the figure it appears that the controls displayed 0% malformations ± ~10 – 12%; however, given the variation, this would require negative values (i.e. a negative malformation rate, which would be biologically implausible) in order to have a zero value with such a large confidence interval. There appears to be some inconsistency with the presented data, since it is not normalized to controls. Moreover, the authors did not indicate whether clutches were screened for malformations prior to the onset of experimentation in order to remove compromised individuals and effectively normalize all treatments. Given that amphibian research generally indicates a malformation rate of ~10% depending on the species, the controls would be expected to have a baseline malformation rate. However, a zero malformation rate in the controls suggests pre-screening, although the variation around the presented zero malformation rate suggests the contrary. In order to achieve the control variation indicated by the authors a malformation rate of >10% would be expected.

2. Relevance of study: **Not relevant**

Comment: Aspects of the experimental design and data analysis and interpretation convey significant uncertainty surrounding the study results reported and question the conclusions attributing exposure to glyphosate formulations with the effects characterized. Furthermore, the duration and magnitude of exposure to glyphosate formulations that occurred in this study greatly exceeds the duration and magnitude of realistic chronic exposures in the environment, detracting from the relevance of the research.

Not tested with the lead formulation under evaluation

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

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Author(s)	Year	Study title
Jayawardena, U.A., Nararatne, A.N., Amerasinghe, P.H., Rajakaruna, R.S.	2011	Acute and chronic toxicity of four commonly used agricultural pesticides on the Asian common toad, *Bufo melanostictus* Schneider. Journal of the National Science Foundation Sri Lanka Volume: 39 Isssue: 3 Pages: 267-276 ISSN: 1391-4588

Abstract[36]

Laboratory and field studies provide evidence that pesticides may play a role in population declines, range reductions and species extinctions of amphibians. The present study examined the acute and chronic toxicity of four commonly used agricultural pesticides, chlorpyrifos, dimethoate, glyphosate and propanil on the survival, growth and development of malformations in the Asian common toad, *Bufo melanostictus*, under laboratory conditions. The 48 hour LC_{50} values of the chemicals were within the Pesticide Area Network (PAN) specified limits, except for propanil, which was less than the PAN specified value. Acute exposure to high concentrations of propanil may have a high direct toxic effect on the Asian common toad. The survival of the tadpoles with chronic exposure to ecologically relevant doses of the four pesticides was significantly reduced (survival in chlorpyrifos 39%, dimethoate 41%, glyphosate 36% and propanil 40% in the highest concentration) than in the control group (93%). Exposed tadpoles took more time to metamorphose but were larger in size than the control tadpoles. They also developed malformations at high frequencies (chlorpyrifos 30%, dimethoate 25%, glyphosate 35% and propanil 15% in the highest concentration). Malformations were mainly axial, including kyphosis (hunched back) and scoliosis (curvature) while skin ulcers and oedemas were also observed. Severe limb malformations such as extra or missing limbs as reported for other species of amphibians exposed to pesticides were not observed in the Asian common toad. None of the tadpoles in the control group had any malformations. Glyphosate exposed metamorphs recorded the highest mortality and malformations at high concentrations (1.0 ppm). However, a profound toxic effect was observed in chlorpyrifos exposed group even at low concentrations (0.1 ppm). The study shows that exposure to commonly used agrochemicals poses serious risk to amphibians in Sri Lanka and highlights the importance of investigating the level of agricultural pesticides in freshwater ecosystems and their effect on non-target organisms.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup® or Glyphosate®
Surfactant:	Speculated by the authors to be polyoxyethylene amine (POEA)
Description:	Roundup® or Glyphosate® is for use in coconut plantations at 1.44 kg/ha in Sri Lanka.
Source of test substance:	Pesticide Registrar's Office, Peradeniya
Lot/Batch #:	Not stated
Purity:	Not stated

[36] Quoted from article

Stock solution: Not stated

2. Vehicle and/or positive control: Not stated

3. Test organism:

Species: *Bufo melanostictus* (Anura, Bufonidae)

Age of test organisms at study initiation: 5 days after hatching (ie. Gosner stage 25-26)

Source: Field-collected from Peradeniya University Park

Holding conditions prior to test: Eggs were kept in glass with dechlorinated tap water and fed with commercial fish feed.

Acclimatisation: none

4. Test system:

Study type: Static (acute) and static renewal (chronic)

Guideline: None

GLP: No

Duration of study: 48 h (acute), and ≥ 30 days (chronic; until metamorphosis)

Test conditions: Glyphosate was tested amongst other pesticides but in separate treatments. The product diluted in dechlorinated water was exposed directly to tadpoles in glass tanks (15 x 15 x 25 cm) containing 2 L test solution. In chronic study, water was renewed every week and tadpoles were fed with commercial fish feed.
In acute studies, mortality was assessed after 48 h. In the chronic study, malformations were recorded at 10 d and 30 d post-hatch (Gosner 27 and 31) and at metamorphosis.

Treatments: 5 concentrations and untreated water control (dechlorinated tap water).

Test concentrations: Acute: 9.50, 11.25, 15.00, 18.75 and 25.0 ppm (based on active ingredient).
Chronic: 0.25, 0.50, 0.75 and 1.00 ppm (a.s.)

Replicates per concentration/control: 3 tanks (tadpoles from 3 different clutches)

Organisms per replicate: 20 per tank

Feeding during experiments: Only in chronic studies with commercial fish feed.

Parameters measured: Acute toxicity: 48-h mortality. LC_{10}, LC_{50} and LC_{90} by probit analysis.
Chronic toxicity: Malformations of snout-vent length (SVL), time required until forelimb emergence of half of tadpoles (TE_{50}).

Analytical determination of test concentrations: None

Validity criteria: Not stated

5. Environmental conditions:

Test medium: Dechlorinated tap water

Temperature: 27-31°C

Photoperiod during tests: 12:12 h

pH: Not stated

Oxygen saturation Not stated

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Composition: Not stated
Hardness: Not stated
Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment

- Study does not match basic scientific principles and is only vague with respect to material & methods.
- Test item not clearly identified – just the trade names of the commercially available products are given without even specification of the product that was indeed used for the study.
- The used fish feed is insufficiently provided.
- Material and methods are widely not standardized and vaguely reported.
- No details about water quality given (ie. pH, oxygen saturation, etc.).
- No analytics to actual test concentrations.

2. Relevance of study:

Comment: **Not relevant**
Not relevant due to weakness in experimental procedure.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

Extended comments:

The authors describe acute and chronic toxicity exposures with the common hourglass tree frog, *Polypedates cruciger*, (a species found in Sri Lanka) using an unknown glyphosate-based formulation described in the paper as simply Roundup® or Glyphosate®. For the glyphosate formulation a relatively high acute LC_{50} value of 45.94 mg/L was reported and this concentration is believed to be expressed in formulation units. The paper also describes chronic exposures to four glyphosate formulation concentrations (0.25, 0.5, 0.75, and 1.0 mg/L) from 5-days post-hatch to metamorphosis. Effects on frog survival and time to front limb emergence for 50% of animals in a treatment group were reported. In addition, malformations including scoliosis, kyphosis, edema and skin ulcers were observed.

Detailed Comments

In the journal article by Jayawardena *et al.*, 2010, the description of the materials, experimental methods and data analysis are inadequate to ensure that the quality of the data obtained is sufficient for use in an ecological risk assessment for amphibians. Major points of the study are:

1) The glyphosate formulation used in testing is referred to by the authors both as “Roundup®” and “Glyphosate®.” It is difficult to evaluate this study because it is not known what was tested.

2) Water quality parameters were not reported. It is indicated that the tadpoles were fed three times per day with commercial fish food, yet the medium was only changed once per week. There is no information on the pH of the medium or the concentration of toxic components such as ammonia.

3) There is insufficient information provided in the paper regarding test conditions and acute and chronic exposure study designs to support the conclusions drawn.

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a) The authors state in the “Chronic exposure to pesticides at ecologically relevant concentrations” section that the exposures were “repeated using tadpoles from different egg clutches separately.” It is unclear if this is reference is to individual replicates or complete duplication of the study.

b) There is no mention of an experimental design. Without a formal experimental design, such as a completely randomized design or randomized complete block design, this experiment can not be considered valid.

4) The statistical treatment and illustration of the results in Figure 1 for the time to 50% emergence (TE_{50}) is misleading and confusing. An F-test (e.g., one way ANOVA) was employed for comparison of treatment mean values; however, a post-hoc statistical analysis (e.g. means separation test) was not performed. Therefore, the authors could not distinguish between a treatment effect and a specific effect at a given concentration level.

5) The calculations to determine the percentage of malformation artificially inflate the number of malformations. The authors divided the number of malformed individuals by the number of surviving individuals in a treatment. The authors should have divided the number of malformed individuals by the starting number of individuals in a treatment to appropriately represent the number of malformations per treatment.

6) The methods of this paper indicate that malformation observations were recorded at 10 days post-hatch, 30 days post-hatch, and then at metamorphosis, yet only 10 days post-hatch data are presented. The authors note that some malformations disappeared with the age of the tadpole. Without data regarding the persistence of malformations through development and information regarding the ability (or inability) of the tadpoles to recover from early injury, it is difficult to weigh the biological significance of the malformations. Given the transitory nature of this observation, this should not be considered to be adverse. Also, the lack of historical control data for this effect in this test system limits the ability to meaningfully interpret this information.

7) The data outlined in Figure 2 pertaining to the incidence of malformation are curious given the variation surrounding the controls. Based on visual interpretation of the figure it appears that the controls displayed 0% malformations ± ~10 – 12%; however, given the variation, this would require negative values (i.e. a negative malformation rate, which would be biologically implausible) in order to have a zero value with such a large a confidence interval. There appears to be some inconsistency with the presented data, since it is not normalized to controls. Moreover, the authors did not indicate whether clutches were screened for malformations prior to the onset of experimentation in order to remove compromised individuals and effectively normalize all treatments. Given that amphibian research generally indicates a malformation rate of ~10% depending on the species, the controls would be expected to have a baseline malformation rate. However, a zero malformation rate in the controls suggests pre-screening, although the variation around the presented zero malformation rate suggests the contrary. In order to achieve the control variation indicated by the authors a malformation rate of >10% would be expected. It should also be noted that another research paper by the same research group focusing on trematode infections in the same species (*Polypedates cruciger*) also indicated a zero frequency of malformation in the control, but no variation (Rajakaruna *et al*. 2008).

8) In addition to the extended static exposure to glyphosate, the crowding in the test aquaria would have created very stressful conditions in these experiments. For static or semi-static conditions, 1.0 g of tissue/L is the OECD criteria for fish, and amphibians are generally more sensitive to ammonia than fish. The average weights of the metamorphs ranged from 0.71 g at the highest glyphosate concentration up to 0.946 g in the control. With 20 organisms per 2L tank, the range of tissue loading by the end of the experiment was 7.1 g/L up to 9.46 g/L, calling into question the validity of the study results. Berger (1968, 1971 as cited by Ouellet in Sparling *et al*. 2000) obtained multiple malformations when tadpoles were reared at densities between 3.5 to 11.1 tadpoles per liter of water.

Conclusions from the review of Jayawardena *et al*. (2011)

Aspects of the experimental design and data analysis and interpretation in Jayawardena *et al.* (2011) convey significant uncertainty surrounding the study results reported and question the conclusions

attributing exposure to glyphosate formulations with the effects characterized. Furthermore, the duration and magnitude of exposure to an unknown glyphosate formulations that occurred in this study greatly exceeds the duration and magnitude of realistic chronic exposures in the environment, greatly diminishing from the relevance of the research. Therefore, the endpoints from this study are not considered to be acceptable for use in an ecological assessment.

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Author(s)	Year	Study title
Jones, D.K., Hammond, J.I., Relyea, R.A.	2010	Roundup® and amphibians: the importance of concentration, application time, and stratification. Environmental Toxicology and Chemistry. Volume: 29 Issue: 9 Pages: 2016-2025 DOI: 10.1002/etc.240 ISSN: 1552-8618 (online)

Abstract[37]

The widespread use of pesticides raises the possibility that non-target organisms might also be affected. To assess this, the traditional approach has been to conduct short-term laboratory experiments spanning a range of lethal concentrations and some longer duration experiments at sublethal concentrations. While this approach has been very useful, less attention has been paid to the timing of exposure and the impacts of multiple, small exposures versus single, large exposures. We examined the role of application amount, timing, and frequency using outdoor mesocosm communities containing larval amphibians (*Rana sylvatica* and *Bufo americanus*) and using a commercial formulation of the herbicide glyphosate (Roundup Original MAX®). Consistent with past studies, exposures of up to 3 mg acid equivalent (a.e.)/L caused substantial amphibian death. However, the amount of death was considerably higher when the herbicide was applied earlier in the experiment than later in the experiment. Single, large applications (at different times) had larger effects on tadpole mortality and growth than multiple, small applications (of the same total amount). The results may reflect an acclimation to the herbicide over time. In treatments with high tadpole mortality, there was no resulting increase in periphyton, suggesting that the reduction in tadpole herbivory might have been offset by direct negative impacts of the herbicide. We also discovered that temperature stratification caused herbicide stratification, with higher concentrations near the surface. Such stratification has important implications to the habitat choices of ectotherms that might prefer surface waters for thermoregulation or prefer deeper waters to avoid predators. Collectively, the present study demonstrates the importance of examining multiple applications times and frequencies to understand the impacts of pesticides on organisms.

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Original MAX®
Active substance(s):	Glyphosate isopropylamine salt (glyphosate-Ipa)
Adjuvant / Surfactant:	Surfactant that is reportedly not POEA (personal communication, S. Mortenson, Monsanto)
Description:	General information about glyphosate background is described in article.
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	48.7% active ingredient
Stock solution:	Herbicide was dissolved in half-liter container of water and subsequently drizzled across mesocosm surface at treatment.

2. Vehicle and/or positive control: Not applicable

[37] Quoted from article

3. Test organism:

Species:	*Rana sylvatica* (Ranidae; wood frog) *Bufo americanus* (Bufonidae; American toad)
Age of test organisms at study initiation:	*Rana sylvatica*: Gosner stage 26 *Bufo americanus*: Gosner stage 25
Source:	Collected as newly oviposited egg masses from natural ponds in the vicinity of University of Pittsburgh.
Handling of species prior to experiment:	Eggs hatched in aged well water and hatchlings were fed rabbit chow ad libitum.
Inoculation of mesocosm:	2 weeks prior to experiment, 200 g of deciduous leaves (*Quercus* spp.), 15 g of rabbit chow and pond water containing zooplankton, phytoplankton and periphyton were added. Mesocosms were covered with 60% shade cloth lids to prevent other organisms from ovipositing. 20 tadpoles of each species were added to every tank.

4. Test system:

Study type:	Mesocosm
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	18 days
Test conditions:	Experimental units were 750-L cattle watering tanks filled with 542 L of well water. Addition of the 2 test species was defined as day 0.
Treatments:	Total of 12 treatments including 9 treatments with 3 different concentrations of single applications on day 0, on day 7 and on day 14, respectively, 2 treatments with 2 concentrations of multiple applications on day 0, 7 and 14, and 1 control treatment.
Test concentrations:	1 x 1 mg a.e./L, 1 x 2 mg a.e./L, 1 x 3 mg a.e./L, 3 x 0.33 mg a.e./L and 3 x 1 mg a.e./L.
Replicates per treatment:	4
Organisms per replicate:	20 tadpoles of each of species in every mesocosm.
Feeding during experiments:	No additional feeding
Parameters measured:	Survival and individual mean mass on day 18 (termination of experiment)
Verification of test concentrations:	Actual concentration of glyphosate was analysed for top and bottom of the water column on days of application after treatment.
Validity criteria:	None

5. Environmental conditions:

Test medium:	Well water
Temperature:	23 – 26°C (measurements on day 7 and day 14)
Photoperiod/Light intensity:	Natural light with 60% shade cloth covering mesocosm tanks (April – May in Pittsburgh, USA)
pH:	7.3-8.2 (measurements on day 7 and day 14)
Dissolved oxygen:	5 - 6.5 mg/L on day 7 and 4 – 8 mg/L on day 14

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Periphyton: Biomass increased over time across all treatments without significant effect among treatments.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions.**

Comment:

- Exposures were conducted in cattle tanks and water depth was not reported.
- Cattle tanks did not contain sediment which is known to allow glyphosate and surfactants to rapidly dissipate from the water column (Wang 2004 et al.). Addition of soil is common practice for microcosm studies.
- Tanks were shaded by cloth lid while biomass of periphyton was still observed to increase indicating no major impact of lower light intensity on mesocosm.
- Effects observed at nominal concentrations of 1, 2 and 3 mg a.e./L are comparable to known responses to amphibians to this formulation and comparable to values reported for fish. No significant effect on survival was noted at 1 mg a.e./L which significantly exceeds an environmentally realistic exposure concentration.
- Some of the reported LC_{50} and all of the LC_{90} values are extrapolated and exceed the highest concentration tested.
- The duration of exposure greatly exceed an environmentally relevant duration and glyphosate concentrations were only measured at the start of the study and not throughout the study.

2. Relevance of study: **Relevant with restrictions**

Comment: A NOEC of 1 mg a.e./L is relevant as it demonstrates no adverse effect at a concentration that exceeds an environmentally realistic exposure concentration..

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
Jones, D.K., Hammond, J.I., Relyea, R.A.	2011	Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. Environmental Toxicology and Chemistry. Volume: 30 Issue: 2 Pages: 446-454 DOI: 10.1002/etc.384 ISSN: 1552-8618 (online)

Abstract[38]

Toxicity assessments on nontarget organisms have largely been addressed using short-term, single-species laboratory experiments. Although extremely helpful, these experiments inherently lack many pervasive ecological stressors found in nature. Though a substantial challenge, incorporating these ecological stressors in contaminant studies would shed light on potential synergistic effects. For the world's leading herbicide, glyphosate, we know little about how natural stressors affect the toxicity to nontarget organisms. To explore how the natural stress of competition might interact with a glyphosate-based herbicide, we used outdoor mesocosms containing three tadpole species that were exposed to a factorial combination of three glyphosate concentrations (0, 1, 2, or 3mg acid equivalent (a.e.)/L of the commercial formulation Roundup Original MAX1) and three tadpole densities (low, medium, or high). We found that increased tadpole density caused declines in tadpole growth, but also made the herbicide significantly more lethal to one species. Whereas the median lethal concentration (LC50) values were similar across all densities for gray tree frogs (*Hyla versicolor*; 1.7–2.3 mg a.e./L) and green frogs (*Rana clamitans*; 2.2–2.6 mg a.e./L), the LC50 values for bullfrogs (*R. catesbeiana*) were 2.1 to 2.2 mg a.e./L at low and medium densities, but declined to 1.6 mg a.e./L at high densities. The large decrease in amphibian survival with increased herbicide concentration was associated with increases in periphyton abundance. We also found evidence that temperature stratification lead to herbicide stratification in the water column, confirming the results of a previous study and raising important questions about exposure risk in natural systems.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Original MAX®
Active substance(s):	Glyphosate isopropylamine salt (glyphosate-Ipa)
Adjuvant / Surfactant:	Surfactant that is reportedly not POEA (personal communication, S. Mortenson, Monsanto)
Description:	General information about glyphosate background is described in article.
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	48.7% active ingredient
Stock solution:	Herbicide was dissolved in half-liter container of water and subsequently drizzled across mesocosm surface at treatment.

2. Vehicle and/or positive control: Not applicable

3. Test organism:

Species:	*Rana catesbeiana* (bullfrog)
	Rana clamitans (green frog)

[38] Quoted from article

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	Hyla versicolor (gray tree frog)
Age of test organisms at study initiation:	Early stage appr. Gosner 25
Source:	Collected as newly oviposited eggs from natural ponds in the vicinity of University of Pittsburgh.
Handling of species prior to experiment:	Eggs hatched in aged well water and hatchlings were fed rabbit chow ad libitum. Tadpoles for experiments were taken from 5-15 different clutches per test species. 20 tadpoles per species were set aside and assessed for 24-h survival due to handling revealing 100% survival.
Inoculation of mesocosm:	2 weeks prior to experiment, 200 g of deciduous leaves (*Quercus* spp.), 15 g of rabbit chow and pond water containing zooplankton, phytoplankton and periphyton were added. Mesocosms were covered with 60% shade cloth lids to prevent other organisms from ovipositing. Tadpoles of each species were added to every tank according to treatments.

4. Test system:

Study type:	Mesocosm
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	22-23 days
Test conditions:	Experimental units were 750-L cattle watering tanks filled with 562 L of well water.
Treatments:	Total of 12 treatments including 3 different concentrations of single glyphosate applications and 1 untreated control crossed with 3 tadpole densities (low, medium, or high).
Test concentrations:	1, 2 and 3 mg a.e./L glyphosate (a.e. = acid equivalent).
Replicates per treatment:	2
Organisms per replicate:	Depending on density treatment: Low density: 20 of each of the three species Medium: 40 of green and gray tree frog and 20 of bullfrog High: 60 of green and gray tree frog and 20 of bullfrog
Feeding during experiments:	No additional feeding
Parameters measured:	Survival and individual mean mass at termination of experiment
Verification of test concentrations:	Actual concentration of glyphosate was analyzed for top and bottom of the water column 3 hours after glyphosate application by using HPLC.
Validity criteria:	None

5. Environmental conditions:

Test medium:	Well water
Temperature:	23 – 26°C (measurements on day 7 and day 16)
Photoperiod/Light intensity:	Natural light with 60% shade cloth covering mesocosm tanks (April – May in Pittsburgh, USA)
pH:	7.3-8.2 (measurements on day 7 and day 14)
Dissolved oxygen:	5 - 6.5 mg/L on day 7 and 4 – 8 mg/L on day 14

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Periphyton: Biomass increased over time across all treatments while significantly more periphyton was found in 3 mg a.e./L treatment..

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions.**

Comment:

- Acceptable, well-documented publication which meets basic scientific principles.
- Tanks were shaded by cloth lid with rather low impact on mesocosm as periphyton was still observed to grow during experimental time. Question arises with respect to significantly more periphyton in the highest concentration of herbicides.
- Water column of highest concentration was not analysed for actual concentrations as it broke during shipping. If shipped together in one package other columns could be contaminated by this accident whereas it is plausibly shown that analytical results are within expectations compared to other cited studies.

2. Relevance of study: **Relevant with restrictions**

Comment: Technically well-concerved experiments comparable to regulatory requirements whereas not under GLP conditions. A restricted reliability can be concluded from broken water sample for analytical verification.

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
King, J.J., Wagner, R.S.	2010	Toxic effects of the herbicide Roundup® Regular on Pacific Northwestern amphibians Northwestern Naturalist Volume: 91 Issue: 3 Pages: 318-324 url: http://www.bioone.org/ doi/abs/10.1898 /NWN09-25.1?journalCode=nwnt DOI: 10.1898/NWN09-25.1 ISSN: 1051-1733 (Print) 1938-5315 (Online)

Abstract[39]**:**

One of the most widely used herbicides for commercial and home use is glyphosate, the active ingredient in Roundup® Regular. We examined toxicity of the herbicide Roundup® on 6 amphibian species: *Ambystoma gracile*, *Ambystoma macrodactylum*, *Anaxyrus* [*Bufo*] *boreas*, *Pseudacris regilla*, *Rana cascadae*, and *Rana luteiventris*. Larvae were exposed to 6 different Roundup® Regular treatments (0 (control), 0.1, 0.5, 1.0, 2.0, and 5.0 mg AI/L dilutions of glyphosate) and monitored for 16 d. Estimated acute lethal concentrations at 24 h (LC50) varied significantly among species (ANOVA, $F_{(3, 56)} \geq = \geq 3.54$, $p < 0.0202$), with concentrations ranging from 0.43 mg AI/L of Roundup® for *P. regilla* to 2.66 mg AI/L for *A. boreas*. Bufonid and ambystomatid larvae were less sensitive than Ranid and Pseudacrid species tested, with no salamander larval mortality occurring at 24 h. Mean time-to-death varied from 1 d for *P. regilla* to 8.3 d for *A. gracile*, respectively (ANOVA, $F_{(5, 971)} \geq = \geq 108$, $p < 0.0001$). For exposure times longer than 24 h, the *A. boreas* was not significantly different than the salamanders for time-to-death, based on Tukey-Kramer comparisons. Results suggest Roundup® Regular is highly toxic to the amphibians at levels below EPA standards for drinking water and at concentrations they may be exposed to during overspray. We recommend the use of less toxic glyphosate-based herbicides in aquatic systems, if applications are necessary, or made during times of year when amphibian larvae are not present.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup Regular®
Active substance(s):	Glyphosate isopropylamine salt
Surfactant:	Not stated
Description:	none
Source of test substance:	Monsanto Company
Lot/Batch #:	Not stated
Purity:	50.2% glyphosate IPA salt
Stock solution:	Stock solution was prepared in a 5 L container and analysed by HPLC.

2. Vehicle and/or positive control: none

3. Test organism:

Species:	Long-toed salamander (*Ambystoma macrodactylum*) Northwestern salamander (*Ambystoma gracile*) Western toad (*Anaxyrus boreas*)

[39] Quoted from article

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Pacific tree frog (*Pseudacris regilla*)
Cascades frog (*Rana cascadae*)
Columbia spotted frog (*Rana luteiventris*)

Age of test organisms at study initiation: Tadpoles 24 h old

Source: Embryos were collected at different localities throughout Kittitas County, Washington, during spring 2005

Holding conditions prior to test: Embryos were kept in separate glass aquaria for each species, aerated in pond water until 24 h after hatching.

Acclimatisation: 24 h

4. Test system:

Study type: Acute static

Guideline: Not stated

GLP: No

Guideline deviations: Not applicable

Duration of study: 16 d

Test conditions: Larvae were exposed in 250 mL beakers filled with 150 mL treatment solution.

Replicates per concentration: 3

Organisms per replicate: 10

Feeding: Not stated

Parameters measured: Survival was monitored daily compared among species by analysing mean time-to-death and LC_{50}. Data were analysed by ANOVA followed by Tukey-Kramer multiple comparisons test, LC_{50} values were determined using trimmed Spearman-Karber method.

Test concentrations: 0, 0.1, 0.5, 1.0, 2.0 and 5.0 mg a.s./L

Analytical determination of test concentrations: HPLC

Validity criteria: Not applicable

5. Environmental conditions:

Test medium: Spring water

Temperature: 18 °C

Photoperiod: 12 h

Light intensity: Not stated

pH: Not stated

Oxygen saturation: Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (major omissions of experimental details)

- Formulation used is not specified further in terms of surfactants.
- pH and oxygen saturation data missing
- Loading information (biomass/L) was not reported.
- Glyphosate concentrations were measured in stock

solutions but not in the exposure tanks.

2. Relevance of study: **Not relevant**

Testing performed with unknown formulation and important experimental details missing. Monsanto does not even sell a formulation named Roundup Regular. This is stated to be a lawn and garden formulation and may contain another active ingredient to promote efficacy and symptomology.

3. Klimisch code: **3 and this study is not adequate for risk assessment**

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Author(s)	Year	Study title
Lajmanovich, R.C., Sandoval, M.T., Peltzer, P.M.	2003	Induction of Mortality and Malformation in *Scinax nasicus* Tadpoles Exposed to Glyphosate Formulations. Bulletin of environmental contamination and toxicology Volume: 70 Number: 3 Pages: 612-618 DOI: 10.1007/s00128-003-0029x ISSN: 1051-0761

Executive summary[40]

In the current study, tadpoles of *Scinax nascius* were exposed under laboratory conditions to GLYFOS®, a formulation containing glyphosate at nominal test concentrations of 3.07, 3.84, 4.8, 6 and 7.5 mg formulation/L. A negative control (artificial pond water) was prepared in parallel. Ten tadpoles were exposed in three replicates in the control and at each treatment level.

All tadpoles were observed at daily intervals for the 96 hour study duration with mortality recorded. At the end of exposure, surviving tadpoles were fixed in formalin solution and examined for morphological changes. Larval malformations were minimal at 3.07 mg/L when tadpoles were exposed for one day, whereas an increased malformation was observed at levels of 7.5 mg Glyfos®/L.

The 96 hour LC_{50} value for tadpoles of *Scinax nascius* exposed to Glyfos® was 2.64 mg formulation/L (nominal) with 95% confidence interval of 2.19 to 2.84 mg/L.

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MATERIALS AND METHODS

1. Test material:

Test item:	Glyphos ®
Active substance(s):	Glyphosate IPA salt
Adjuvant / Surfactant:	POEA, according to authors but can not be confirmed
Source:	Not reported
Purity:	48% glyphosate as isopropylamine salt
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test organism:

Species:	*Scinax nasicus*
Stages of test animals at collection:	Gosner stage 18-24
Stages of test animals at study initiation:	Gosner stage 25-26 (prometamorphic larvae)
Sex:	Not reported
Source:	Temporary pond in the Floodrain Parana River (31°42’S; 60°34’O, Parana, Argentina)
Body weight:	0.01 ± 0.001 g

[40] No abstract in article, compiled by DKC

Acclimation period: 7 days

Handling of species prior to experiment: Tadpoles were housed in glass tanks with artificial pond water (APW) of pH 6.8, conductivity 149 µmhos/cm^{-1}, dissolved oxygen 5.5 ± 1 mg/L, hardness 66.6 mg/L of $CaCO_3$ at 22 ± 2°C and a 12 hour light/dark cycle.

Feeding: Not reported

4. Test system:

Study type: Semi-static

Guideline: US EPA (1975) Methods for acute toxicity test with fish, macroinvertebrate and amphibians. Ecol Res Ser. EPA-660/3-75-009;

US EPA (1989) Short term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd Edition.

GLP: No

Guideline deviations: Environmental adaptations to the pond water; feeding not reported; no weight and length measurement carried out.

Duration of study: 96h

Test conditions: Glass tank (35 cm diameter and 60 cm high), filled with 4 L of APW. Control and test solutions were renewed daily

Test concentrations: Control and 3.07, 3.84, 4.8, 6, and 7.5 mg of glyphosate formulation

Replicates per treatment: 3

Organisms per replicate: 10 tadpoles per tank

Feeding during treatment: Not stated

Parameters measured: Survival was recorded every 24 h, external morphology was examined with binocular microscopy after the end of exposure. The LC_{50} with confidence limits ($p \leq 0.05$) were estimated by using an analysis program based on Finney (1971). Data from control and experimental groups were analysed by one-way analysis of variance in conjunction with LSD test.

5. Environmental conditions:

Test medium: Artificial pond water (APW)

Temperature: 22 ± 2°C

Photoperiod/Light intensity: 12 h

pH: Not stated

Dissolved oxygen: Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:

- Study carried out according to US EPA guidelines on the relevant study methods. Minor adaptations done in the studies (environmental adaptations to the pond water).
- Major reporting deficiencies (feeding not reported; no weight and length measurement carried out), no pH, no

oxygen measurements.

- No analytical verification conducted.

2. Relevance of study: **Relevant wth restrictions**

Testing performed with unknown formulation and no analytical verification conducted. Concentrations resulting in adverse effects only occurred at concentrations that greatly exceeded environmentally realistic exposures. Effects were at background levels at the tested concentration of 3.07 mg formulation/L (2% mortality and 5% malformations), which exceeds an environmentally realistic exposure level by x-fold. However, control mortality exceeded this level and the background malformation level was not reported but was likely comparable. Malformations were only observed at levels that greatly exceeded environmentally realistic exposures and that resulted in significant mortality.

3. Klimisch code: **3 and this study is not adequate for risk assessment**

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Author(s)	Year	Study title
Lajmanovich, R., Lorenzatti, E., Maitre, M.I., Enrique, S., Peltzer, P.	2003	Comparative acute toxicity of the commercial herbicides glyphosate to neotropical tadpoles *Scinax nasicus* (Anura : Hylidae) Fresenius Environmental Bulletin Volume: 12 Issue: 4 Pages: 364-367 DOI: not available ISSN: 1018-4619

Abstract[41]

We investigated the effect of glyphosate (GLY) to *Scinax nasicus* tadpoles and the influence of GLY degradations in the variation of LC_{50} values. These results showed that 96-h LC_{50} for *S. nasicus* tadpoles exposed to continuous applications of GLY in the renewal tests (RT) was 3.13 mg GLY/L, compared to an estimated LC_{50} in static tests (ST) of 5.27 mg GLY/L. These data indicate large differences in toxicity between RT and ST, *S. nasicus* tadpoles did not die when the GLY exposure was not continuous.

MATERIALS AND METHODS

1. Test material:

Test item: Glyfos®

Active substance(s): Glyphosate

Adjuvant / Surfactant: Stated to be POEA but cannot confirm

Description: Not stated

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: 48 % glyphosate, measured by high performance liquid chromatography (HPLC)

Stock solution: Not stated

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Scinax nasicus* tadpoles

Age/ stage/ weight of test organisms at study initiation: Gosner Stage 26-27, average weight: 0.025 ± SE 0.006 g

Source: Collected from an unpolluted temporary pond in the floodplain of the Paraná River (31º 42´S; 60º 34´O, Santa Fe, Argentina),

Holding conditions prior to test: Gosner Stage 25-26, 12 h light/12 h dark cycles in glass tanks with artificial pond water (APW) (pH: 8.2 ± 0.3, conductivity: 237 ± 25 µmhos/cm^{-1}, dissolved oxygen: 8.4 ± 0.1 mg/L, and hardness: 56 ± 12 mg $CaCO_3$/l, at 22 ± 2 ºC).

Acclimatisation: 7d

4. Test system:

Study type: laboratory, renewal and static test

Guideline: ASTM (1993): Standard practice for conducting acute tests

[41] Quoted from article

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with fishes, macroinvertebrates and amphibians. Designation E 729-88a. In: ASTM standards on aquatic toxicology and hazard evaluation. Philadelphia, PA, pp. 102-121.

GLP: Not stated

Guideline deviations:
- The species *Scinax nasicus* is used instead of the recommended species *Rana* sp or *Bufo* sp.
- A light dark cycle of 12:12 h instead of an 16:8 h cycle was chosen
- The experiment was carried out with an dilution factor of 1.25 instead of an dilution factor of 0.6 (but there was no range finding test stated)
- No observation was conducted after 3, 6 and 12 h to count dead animals as proposed

Duration of study: 96 h

Test conditions: Glass tanks (35 cm diameter and 60 cm high), with 4 L of artificial pond water

Treatments: 5

Test concentrations: 0, 3.07, 3.84, 4.8, 6.0, 7.5 mg GLY-F/L

Replicates per treatment: Not stated

Organisms per replicate: 10 tadpoles per tank (loading = 0,063 - 0,078 g/L)

Feeding during experiments: Not stated

Parameters measured: LC_{50}, bobbing (swimming to the surface for air) rates, number of times that larvae broke the water surface with the anterior part of their body

Analytical determination of test concentrations: Water 600 HPLC system with IC-Pack ion-exclusion 50 Å-7µm (7.8 x 150 mm) column, mobile phase phosphoric acid: 0.05% at 55 °C, Water® post column derivatisation system at 38 °C, fluorescence spectrophotometer detector at 339-nm excitation and 345-nm emission wavelengths by Milenium[32] manager data processor. However, analytical results are not reported.

Validity criteria: See guideline

5. Environmental conditions:

Test medium: Artificial pond water

Temperature: 22 ± 2°C

Photoperiod: 12 : 12 h light-dark-cycle

Light intensity: Not stated

pH: 8.2 ± 0.3

Oxygen saturation/ concentration: 8.4 ± 0.1 mg/L (= 96 %)

Conductivity: 237 ± 25 µmhos/cm^{-1}

Hardness: 56 ± 12 mg $CaCO_3$/L

Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **No reliable**

Comment:
- Test procedure is comparable to guideline study

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(deviations are existing but acceptable)

- Unclear, whether endpoint is given as mg active substance/L or mg formulation/L, as both 'versions' are stated in the manuscript.
- Number of replicates not stated.
- No control data presented.
- Analytical results not reported.

2. Relevance of study: **Not relevant**

Comment: This study is based on US national guidelines, however, the documentation is insufficient to verify whether study is valid (e.g., analytical results not reported). Even the lowest concentration tested – although it is not clear whether these refer to active substance or formulation significantly exceeds an environmentally realistic exposure level.

3. Klimisch code: **3 and this study is not adequate for risk assessment**

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Author(s)	Year	Study title
Lajmanovich, R.C., Attademo, A.M., Peltzer, P.M., Junges, C.M., Cabagna, M.C.	2011	Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors Archives of Environmental Contamination and Toxicology Volume: 60 Issue: 4 Pages: 681-689 url: http://www.springerlink.com/content/43282w6235j23273/ DOI: 10.1007/s00244-010-9578-2 ISSN: 0090-4341 (Print) 1432-0703 (Online)

Abstract[42]

In this study, amphibian tadpoles *Rhinella arenarum* were exposed to different concentrations of Roundup Ultra-Max (ULT), Infosato (INF), Glifoglex, and C-K YUYOS FAV. Tadpoles were exposed to these commercial formulations with glyphosate (CF-GLY) at the following concentrations (acid equivalent [ae]): 0 (control), 1.85, 3.75, 7.5, 15, 30, 60, 120, and 240 mg ae/L for 6–48 h (short-term). Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CbE), and glutathione *S*-transferase (GST) activities were measured among tadpoles sampled from those treatments that displayed survival rates >85%. Forty-eight-hour LC_{50} for *R. arenarum* tadpoles exposed to CF-GLY in the static tests ranged from ULT = 2.42 to FAV = 77.52 mg ae/L. For all CF-GLY, the LC_{50} values stabilized at 24 h of exposure. Tadpoles exposed to all CF-GLY concentrations at 48 h showed decreases in the activities of AChE (control = 17.50 ± 2.23 nmol/min/mg/protein; maximum inhibition INF 30 mg ae/L, 71.52%), BChE (control = 6.31 ± 0.86 nmol/min/mg/protein; maximum inhibition INF 15 mg ae/L, 78.84%), CbE (control = 4.39 ± 0.46 nmol/min/mg/protein; maximum inhibition INF 15 mg ae/L, 81.18%), and GST (control = 4.86 ± 0.49 nmol/min/mg/protein; maximum inhibition INF 1.87 mg ae/L, 86.12%). These results indicate that CF-GLY produce a wide range of toxicities and that all enzymatic parameters tested may be good early indicators of herbicide contamination in *R. arenarum* tadpoles.

MATERIALS AND METHODS

1. Test material:

Test item(s): Roundup Ultramax®
Infosato
Glifoglex
C-K Yuyos FAV

Active substance(s): Glyphosate isopropylamine salt

Surfactant: Unspecified inert surfactant, that does not require testing according to authors

Description: none

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: Roundup Ultra®: commercial grade, 74.7% a.i.
Infosato, Glifoglex, C-K Yuyos FAV: commercial grade, 48% a.i., each

[42] Quoted from article

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Stock solution: Not stated

2. Vehicle and/or positive control: none

3. Test organism:

Species: *Rhinella arenarum*

Age of test organisms at study initiation: Tadpoles (prometamorphic), Gosner stage 36-38

Size: 0.93 ± 0.09 mm and 0.27 ± 0.08 g

Source: Larvae were collected in December 2009 from temporary ponds in non-agricultural areas in natural parafluvial forests of the Paraná River Boundary, Argentina.

Holding conditions prior to test: Not stated

Acclimatisation: Not stated

4. Test system:

Study type: Acute static

Guideline: Not stated

GLP: No

Guideline deviations: Not applicable

Duration of study: 48

Test conditions: Larvae were exposed in glass tanks (12.5 cm diameter × 13.5 cm height) filled with 1 L of DTW (deionised tap water?). Whole tadpoles were homogenized in 0.1% triton X-100, 25 mM Tris-HCL (pH 8.0)

Replicates per concentration: 3

Organisms per replicate: 7

Feeding: None during test

Parameters measured: Survival after 6, 12, 24 and 48 h, enzyme activities. AChE and BChE were measured according to Ellman et al. 1961 at 410 nm, protein concentrations in the supernatant were determined using the Biuret method. CbE activities were also determined spectrophotometrically using α-NA (530 nm) as substrate. GST activity was determined at 340 nm in Na-phosphate buffer, CDNB and GSH.
LC_{50} values and its confidence limits were determined by trimmed Spearman-Karber. LC_{50} values were compared using one-way ANOVA followed by Duncan's Multiple Range test as post-hoc. Enzymatic activity results were tested for differences using two –way ANOVA using GLMs and Dunnett's test for post-hoc comparisons.

Test concentrations: 1.85, 3.75, 7.5, 15, 30, 60, 120 and 240 mg a.e./L, for each formulation and DTW control

Analytical determination of test concentrations: none

Validity criteria: Not applicable

5. Environmental conditions:

Test medium: DTW – abbreviation not specified, probably deionised tap water?

Temperature: 22 ± 2 °C

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Photoperiod:	12 h
Light intensity	Not stated
pH:	Not stated
Oxygen saturation	Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (major omissions of experimental details)

- It is cited that POEA might have a significant effect on amphibians, but for the experimental setup surfactants are considered as inert ingredients which do not require testing?
- Formulations used are not specified further.
- Not clear how test units were determined, i.e. how amount of formulation transfers to a.e., when no analytical verification was conducted.
- pH and oxygen saturation data missing

2. Relevance of study: **Not relevant**
Testing performed with unknown formulations.

3. Klimisch code: **3 and this study is not adequate for risk assessment**

Extended comments

In this paper, tadpoles were treated at exposure levels that greatly exceeded predicted exposure concentrations to several glyphosate based formulations and examined the effect of acute exposure to acetylcholinesterase, butyrylcholinesterase, carboxylesterase, and glutathione S-transferase activities in tadpoles from treatments that had >85% survival. The use of the >85% survival criterion is clearly not the case. For example in the Roundup Ultra treatments, enzyme measurements were made on tadpoles exposed to 1.85 and 3.75 mg glyphosate acid equivalents/L for 48 hours. However, the 48 hour LC_{50} (median lethal concentration) was reported to be 2.42 mg glyphosate acid equivalents/L with the 95% confidence interval ranging from 2.19 to 2.75 mg glyphosate acid equivalents/L. Consequently, these sub-lethal measures were made on organisms from treatments that were likely experiencing significant lethality. It is surprising that this point was not questioned in the peer-review process prior to publication. Additionally, in the activity measurements, only 1 out of 16 assays (4 formulations X 4 enzymes) showed a trend toward dose dependence. To not consistently demonstrate a dose response is uncharacteristic of a response for these enzyme systems and questions the validity of this data. The authors describe glyphosate an organophosphate pesticide that inhibits cholinesterase activity through a well defined toxicological mechanism. Glyphosate is not an organophosphate pesticide and the rationale they refer to in the paper for cholinesterase inhibition is incorrect. Based on the review of this paper, it is evident that all enzyme activity evaluations were confounded by exposure to unrealistically high exposure concentrations and these results do not reflect what would occur under field exposure conditions.

Author(s)	Year	Study title
Piha, H., Pekkonen, M., Merila, J.	2006	Morphological Abnormalities in Amphibians in Agricultural Habitats: A Case Study of the Common Frog *Rana temporaria* Copeia Volume: 4 Pages: 810-817 ISSN: 0045-8511

Abstract[43]

Recent studies suggest that the incidence of morphological abnormalities has increased in many amphibian populations, often exceeding the estimated background deformity frequency of 0–5%. Many chemical contaminants, including agrochemicals, can cause abnormalities in amphibians, but data on the occurrence of morphological abnormalities in wild amphibian populations in Europe is anecdotal at best. In a large scale study covering 264 ha and 26 farmland breeding populations of the Common frog (*Rana temporaria*) in southern Finland, we investigated whether the incidence of morphological abnormalities in metamorphs differed from the background level of 0–5% and among populations along an agrochemical gradient. Abnormalities occurred in a low frequency (1% of the studied individuals; 40/4115), the highest population specific frequency being 4%. We found no evidence for increased abnormality frequencies in the habitats most likely exposed to agrochemicals. Hence, the data suggest that current Finnish agrochemical practices are not causing increased incidences of morphological abnormalities in Common frog populations breeding in farmland areas.

MATERIALS AND METHODS

1. Test material:

Test item(s): Pesticides in general

Active substance(s): Not stated

Surfactant: Not stated

Description: Among most frequently use pesticides in Finland is glyphosate while it was not detected to occur in higher concentrations.

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: Not stated

Stock solution: None

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Rana temporaria* (Anura, Ranidae)

Age of test organisms at study initiation: Gosner stage 42

Source: Field-collected from natural ponds in agriculturally intensive region of Finland

Holding conditions prior to examination: Kept in 10-L buckets and stored in a cold room

Acclimatisation: Not applicable

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[43] Quoted from article

4. Test system:

Study type:	Field survey
Guideline:	None
GLP:	No
Duration of study:	Not applicable (exposure to uncertain amount of pesticides is supposed to happen in the field; this study is only an examination of tadpoles)
Test conditions:	None.
Treatments:	Frog populations in intensively agricultural region in Finland: • Plowed field • Agricultural grass • Forest
Test concentrations:	Not determined
Replicates per treatment:	9 in ploughed field, 7 in grassland and 10 in forest (collection from totally 26 breeding sites).
Organisms per breeding site:	Appr. 150 individuals
Parameters measured:	Frequency of external morphological abnormalities.
Analytical determination of test concentrations:	Not specified
Validity criteria:	None

5. Environmental conditions:

Test medium:	Not specified
Weather conditions / season / daylight:	Natural weather conditions during June and July 2002 in southern Finland.

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment

- Expected background frequency of 0-5% not validated and without references.
- No clear definition for the accounted morphological abnormalities while three excluded metamorphs are precisely described.
- Treatment and exposure to quantity and quality of pesticides is extremely vague as well as environmental conditions
- Unclear if collected individuals were ever in contact with pesticides let alone with glyphosate.

2. Relevance of study: **Not relevant**

Comment:

This study investigates the frequency of amphibian abnormalities due to pesticidal use in general. Glyphosate plays only a minor role as it is not among the chemicals which were detected 2-3 years after the collection.

3. Klimisch code: **Klimisch rating of 3.**

Author(s)	Year	Study title
Relyea, R. A.	2004	Growth and survival of five amphibian species exposed to combinations of pesticides. Environmental Toxicology and Chemistry. Volume: 23 Issue: 7 Pages: 1737-1742 DOI: ISSN: 0730-7268 (print), 1552-8618 (online)

Abstract[44]

The global decline of amphibians has sparked interest in the role that pesticides may play. Pesticides in nature typically exist in combinations, but given the vast number of chemicals used, most toxicological experiments necessarily have examined one pesticide at a time. I examined how four commercial formulations of pesticides (diazinon, carbaryl, malathion, and glyphosate) affected the survival and growth of five larval amphibian species (*Rana pipiens, R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*) when alone (at 1 or 2 mg/L of active ingredient) and in pairwise combinations (1 mg/L of each pesticide). At 1 mg/L, the pesticides reduced survival in 5% of the 20 species–pesticide comparisons and reduced growth in 35% of the comparisons. At 2 mg/L, the pesticides had more widespread effects, reducing survival in 35% of the 20 species–pesticide comparisons and reducing growth in 70% of comparisons. Combined pesticides occasionally caused lower survival and growth than either pesticide alone, but the effects were never larger than the more deadly of the two pesticides alone at 2 mg/L. This suggests that the impact of combining these four pesticides is similar to that predicted by the total concentration of pesticides in the system.

MATERIALS AND METHODS

1. Test material:

Test item:	Diazinon, malathion, Sevin® and Roundup®
Active substance(s):	Diazinon, malathion, carbaryl (in Sevin®) and glyphosate (in Roundup®)
Adjuvant / Surfactant:	Only stated for Roundup®, which is reported to contain a POEA surfactant (polyethoxylated tallowamine) but was not analytical verified.
Description:	Diazinon, malathion and carbaryl are commonly used insecticides and glyphosate is the most commonly used herbicide in the United States.
Source of test substance:	Commercial formulations from retailer
Lot/Batch #:	Not stated
Purity:	Purity verified by HPLC: diazinon = 22.4%, malathion = 50.6%, carbaryl = 22.3% and glyphosate = 25.2%
Stock solution:	None, applied to each experimental unit individually.

2. Vehicle and/or positive control: Not stated

3. Test organism:

Species: *Rana pipiens* (Leopard frog)
Rana clamitans (green frog)

[44] Quoted from article

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Rana catesbeiana (bullfrog)
Bufo americanus (American toad)
Hyla versicolor (gray tree frog)

Age of test organisms at study initiation: Gosner stage 25 (after hatching from egg masses)

Source: Field collected from outdoor wading pools; no specific locations are reported

Holding conditions prior to test: None

Acclimatisation: None

4. Test system:

Study type: Laboratory chronic, static renewal test (water was changed and reapplied with pesticides every 4 days)

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 16 days.

Test conditions: Experimental units were 10-L plastic tubs filled with 8 L prepared water and renewed every 4 days. Daily survival assessment.

Treatments: Totally 15 treatments with each of the test species:
4 Pesticides alone at 2 concentrations, and 6 pairwise combinations of four pesticides and 1 untreated control.

Test concentrations: 1 and 2 mg/L for pesticides alone and 2 mg/L for pairwise pesticide combinations (containing 1 mg/L of each pesticide). These nominal test concentrations were based on the analytical identified purity of active ingredients in the used commercial formulations.

Replicates per treatment: 4

Organisms per replicate: 10 tadpoles (from 3-10 different egg masses per species)

Feeding during experiments: Ground fish flakes every 2 days (9% daily per capita ration until midway and then doubled)

Parameters measured: Survival

Analytical determination of test concentrations: None

Validity criteria: None

5. Environmental conditions:

Test medium: Charcoal-filtered, ultraviolet-irradiated well water.

Water temperature: 20.4 – 20.9 °C

pH: 8.0

Dissolved oxygen saturation 2.8 – 3.5 mg/L

Ammonia: 2.8-4.2 mg/L

Water quality was only measured in experiments with *Rana pipiens* and *Bufo americanus*.

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable.**

Comment:

- Lack of analytical quantification of test concentrations and endpoints are based on nominal not measured concentrations.
- Source of test organisms are insufficiently reported.
- No valid identification reported about test species.
- The glyphosate formulation tested is not clearly identified – just the trade without specification of the product that was used for the study.
- The fish feed is insufficiently described.
- Non standardized testing methodology was used and important experimental details are vaguely reported.
- Statistical results are only given as p-values without test parameters.
- Pooling of results across test species for general conclusions is deemed inadequate after finding very different responses of each test species alone.
- Water quality only measured for 2 test species (*Rana pipiens* and *Bufo americanus*)

2. Relevance of study: **Not relevant**

Comment: Lack of analytical confirmations of test concentrations, , missing details regarding the experimental procedure significantly impacts the validity of the study.

Therefore, the study is judged not to be relevant for risk assessment, the study tested concentrations of glyphosate (1 and 2 mg/L) that greatly exceeded predicted field exposure concentrations and PECsw from FOCUS step 1 of 0.101 mg/L and the duration of exposure 16 d represents a chronic exposure to concentration levels that exceed acute exposure levels.

Study was not performed with the lead formulation MON 52276 under evaluation.

3. Klimisch code: **Klimisch rating of 3**

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Author(s)	Year	Study title
Relyea, R. A.	2005	The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications. Volume: 15 Issue: 2 Pages: 618-627 DOI: 10.1890/03-5342 ISSN: 1051-0761 (online)

Abstract[45]

Pesticides constitute a major anthropogenic addition to natural communities. In aquatic communities, a great majority of pesticide impacts are determined from single species experiments conducted under laboratory conditions. Although this is an essential protocol to rapidly identify the direct impacts of pesticides on organisms, it prevents an assessment of direct and indirect pesticide effects on organisms embedded in their natural ecological contexts. In this study, I examined the impact of four globally common pesticides (two insecticides, carbaryl [Sevin] and malathion; two herbicides, glyphosate [Roundup] and 2,4-D) on the biodiversity of aquatic communities containing algae and 25 species of animals.

Species richness was reduced by 15% with Sevin, 30% with malathion, and 22% with Roundup, whereas 2,4-D had no effect. Both insecticides reduced zooplankton diversity by eliminating cladocerans but not copepods (the latter increased in abundance). The insecticides also reduced the diversity and biomass of predatory insects and had an apparent indirect positive effect on several species of tadpoles, but had no effect on snails. The two herbicides had no effects on zooplankton, insect predators, or snails. Moreover, the herbicide 2,4-D had no effect on tadpoles. However, Roundup completely eliminated two species of tadpoles and nearly exterminated a third species, resulting in a 70% decline in the species richness of tadpoles. This study represents one of the most extensive experimental investigations of pesticide effects on aquatic communities and offers a comprehensive perspective on the impacts of pesticides when nontarget organisms are examined under ecologically relevant conditions.

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (Roundup ®) but particular formulation unknown, likely a concentrated lawn and garden formulation.
Active substance(s):	Glyphosate
Adjuvant / Surfactant:	polyethoxylated tallowamine (POEA) stated in paper but unknown
Description:	none
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	Glyphosate: 25.2 %[46]
Stock solution:	Not stated

2. Vehicle and/or positive control: None

3. Test organism:

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[45] Quoted from article

[46] Confirmed by the Mississippi State Laboratory (Mississippi State, Mississippi, USA)

Species investigated: Diverse mesocosm communities: especially amphibians, zooplankton, snails, backswimmers, diving beetles

Age and size of test organisms at study initiation:

Species	Age and size
Ambystoma maculatum:	49 ± 3 mg
Dytiscus sp.:	28 ± 1,1 mm
Acilius semisulcatus:	21 ± 0,4 mm
Anax junius:	39 ± 0,9 mm
Tramea sp.:	23 ± 0,6 mm
Lestes sp.:	15 ± 0,3 mm
Notonecta undulata:	10 ± 0,3 mm
Belostoma flumineum:	20 ± 0,2 mm
Rana sylvatica:	104 ± 10 mg
Rana pipiens:	42 ± 8 mg
Bufo americanus:	45 ± 5 mg
Hyla versicolor:	4 ± 0 mg
Pseudacris crucifer:	214 ± 16 mg
Physa integra:	62 ± 4 mg
Helisoma trivolvis:	434 ± 31 mg
Stagnicola elodes:	177 ± 20 mg

Source: Not stated

Maturation and inoculation of organisms:
26th -28th April: tanks filled with well water
6th May: Addition of 300 g dry leaves and 25 g rabbit chow for nutrient supply
28th May – 30th May: Addition of macroorganisms (tadpole larval anurans, snails, larval damselflies, dragonflies, larval *Dytiscus* and *Acilius* beetles, *Notonecta* and *Belostoma hemipterans*, salamander larvae

Acclimatisation: Depending on inoculation start point (see bench before)

4. Test system:

Study type: Lentic mesocosm study, randomized design

Guideline: None

GLP: Not stated

Guideline deviations: -

Duration of study: 30th May – 12th June 2002

Test conditions: 1200-L experimental units were filled with 1000 L well water and inoculated with additionally organic matter

Application: Overspray, for each pesticide the maximum in-field application rate according to manufacturer's recommendation based on surface area of ponds.

Application devices: Not stated

Application verification: Not stated

Treatments: 5 pesticides (each at worst-case concentration) and 1 control

Test concentrations: Glyphosate: 3.8 mg/ L

Replicates per treatment: 6

Organisms per replicate: Aquatic pond community

Additional nutrient supply: Nutrient supply with dry leaves and rabbit chow (see above)

Parameters measured: Community diversity, abundance
Taxa survival and biomass.

Analytical determination of test: Not stated

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concentrations:

Validity criteria: Not stated

5. Environmental conditions:

Test medium: Matured well water

Temperature: Environmental conditions

Photoperiod: Not stated

Light intensity: Not stated

pH: Not stated

Oxygen saturation Not stated

Conductivity: Not stated

Hardness: Not stated

Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:

- Duration of the mesocosm study is very short, therefore no recovery of community could be investigated
- No effect calculation was conducted
- methodological details missing (e.g. pond construction, application)
- Considering that a herbicide is applied, information on algae (e.g. community structure or algae biomass) is missing. Algae may provide a primary source of nutrient which is missing after herbicide application.
- Statistics applied for evaluation of species richness are not appropriate; an evaluation using standardised tools such as the Shannon's diversity H′ or the Simpsons Diversity Index is missing.
- In mesocosm studies, a high natural (biological) variability is expected, but nothing is reported here. This will definitely affect statistical evaluation (ANOVA).

2. Relevance of study: **Relevant with restrictions**

Comment: This study tested what is believed to be a concentrated lawn and garden formulation that is not similar to the lead formulation in the glyphosate re-evaluation. This study tested concentrations that greatly exceed realistic levels predicted for surface water. The test concentration for glyphosate was 3.8 mg a.s./L compared to the $PECS_{sw}$ from tier 1 FOCUS of 0.101 mg a.s./L, making the concentration tested in this study nearly to 40 times a worst-case PEC_{sw} and exceeds even a worst case direct overspray which is not an approved use and permitted by the label.

3. Klimisch code: **Klimisch rating of 2**

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Author(s)	Year	Study title
Relyea, R. A.	2005	The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology. Volume: 48 Pages: 351-357 DOI: 10.1007/s00244-004-0086-0 ISSN: 0090-4341 (print), 1432-0703 (online)

Abstract[47]

The decline in amphibians across the globe has sparked a search for the causes, and recent evidence suggests a connection with pesticides. However, for most pesticides, tests on amphibians are rare and conducted only for short durations (1 to 4 days) and without natural stressors. Recent studies have discovered that the stress of predator cues in the water can make insecticides much more lethal to larval amphibians, but it is unknown whether this phenomenon can be generalized to other types of pesticides. Using six species of North American amphibian larvae (*Rana sylvatica*, *R. pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*), I examined the impact of a globally common herbicide (Roundup) on the survival of tadpoles for 16days with and without the chemical cues emitted by predatory newts (*Notophthalmus viridescens*). LC_{50} 16-d estimates varied from 0.55 to 2.52 mg of active ingredient (AI)/L, which was considerably lower than the few previous studies using Roundup (1.5 to 15.5 mg AI/L). Moreover, in one of the six species tested (*R. sylvatica*), the addition of predatory stress made Roundup twice as lethal. This discovery suggests that synergistic interactions between predatory stress and pesticides may indeed be a generalizable phenomenon in amphibians that occurs with a wide variety of pesticides.

MATERIALS AND METHODS

1. Test material:

Test item: Roundup®

Active substance(s): Glyphosate isopropylamine salt (glyphosate-IPA)

Adjuvant / Surfactant: Supposed to contain polyethoxylated tallowamine (POEA)

Description: Commercial form of glyphosate

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: 25.2% glyphosate-IPA verified by HPLC

Stock solution: None

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Rana sylvatica* (wood frog)
Rana pipiens (Leopard frog)
Rana clamitans (green frog)
Rana catesbeiana (bullfrog)
Bufo americanus (American toad)
Hyla versicolor (gray tree frog)

Age of test organisms at study initiation: Gosner stage 25 (after hatching from egg masses)

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[47] Quoted from article

Source: Field-collected as eggs in ponds and marshes from northwestern Pennsylvania (USA).

Holding conditions prior to test: Eggs were hatched in aged well water and kept predator naïve.

Acclimatisation: None

4. Test system:

Study type: Static renewal test (every 4 days renewal)

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 16 days

Test conditions: Experimental units were 10-L plastic tubs containing 7.8 L well water. In each unit 10 tadpoles were exposed to different test concentrations. At each concentration and untreated control, two predator treatments were applied, i.e. predator present or absent. Predator red-spotted newt (*Notophthalmus viridescens*) was added in cages so that emitted chemical cues can diffuse through water and induce prey stress (method validated by previously published studies). Newt cages were screened plastic cups with 1 adult newt for predator presence and empty for predator absence treatment.

Treatments: Totally 12 treatments: predator present or absent crossed with 5 glyphosate test concentrations and 1 non-glyphosate control

Test concentrations: 0.1, 1.0, 5.0, 10.0 and 20.0 g a.s./L

Replicates per treatment: 4

Organisms per replicate: 10

Feeding during experiments: Ground fish flakes at 18% of their body mass per day and doubled in midway of experiment.

Parameters measured: Survival assessed daily

Analytical determination of test concentrations: None

Validity criteria: None

5. Environmental conditions:

Test medium: Charcoal filtered, UV-irradiated well water.

Temperature: Wood frog: 18.5-18.9°C
Leopard frog: 18.6-18.8°C
Green frog: 21.2-21.3°C
Bullfrog: 21.3-21.4°C
American toad: 20.1-20.2°C
Gray tree frog: 20.1-20.3°C

Photoperiod: 14:10 hours photo-/scotophase

Light intensity Laboratory lightning

pH: 7.8-8.3

KLIMISCH EVALUATION

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1. Reliability of study: **Reliable with restrictions**

Comment:
- Lack of analytical quantification of test concentrations decreases the reliability and endpoints can only be based on nominal concentrations.
- No valid identification reported about test species.
- Test item not clearly identified – just the trade names of the commercially available products are given without even specification of the product that was indeed used for the study.
- Information about used fish feed is insufficiently provided.

2. Relevance of study: **Relevant with restrictions**

Comment: The study provides some relevant data while interpretation of results is difficult due to some weakness in experimental procedure, i.e. lack of analytics, insufficient identification of test items and test organisms.

Toxicity values were generated with a formulation other than the lead formulation, MON 52276.

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
Relyea, R.A.	2005	The lethal impact of roundup on aquatic and terrestrial amphibians. Ecological Applications. Volume: 15 Issue: 4 Pages: 1118-1124 DOI: 10.1890/04-1291 ISSN: 1051-0761

Abstract[48]

The global decline in amphibian diversity has become an international environmental problem with a multitude of possible causes. There is evidence that pesticides may play a role, yet few pesticides have been tested on amphibians. For example, Roundup is a globally common herbicide that is conventionally thought to be nonlethal to amphibians. However, Roundup has been tested on few amphibian species, with existing tests conducted mostly under laboratory conditions and on larval amphibians. Recent laboratory studies have indicated that Roundup may be highly lethal to North American tadpoles, but we need to determine whether this effect occurs under more natural conditions and in post-metamorphic amphibians. I assembled communities of three species of North American tadpoles in outdoor pond mesocosms that contained different types of soil (which can absorb the pesticide) and applied Roundup as a direct overspray. After three weeks, Roundup killed 96–100% of larval amphibians (regardless of soil presence). I then exposed three species of juvenile (post-metamorphic) anurans to a direct overspray of Roundup in laboratory containers. After one day, Roundup killed 68–86% of juvenile amphibians. These results suggest that Roundup, a compound designed to kill plants, can cause extremely high rates of mortality to amphibians that could lead to population declines.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® "Weed and Grass Killer" this is believed to be a concentrated lawn and garden formulation
Active substance(s):	Glyphosate isopropylamine salt (glyphosate-IPA)
Adjuvant / Surfactant:	POEA
Description:	Commercially available herbicide for home and garden use against terrestrial and aquatic weeds.
Source of test substance:	Commercially purchased
Lot/Batch #:	Not stated
Purity:	25.2% glyphosate
Stock solution:	None

2. Vehicle and/or positive control: Not applicable

3. Test organism:

Species:	Aquatic experiments: *Rana pipiens* (leopard frog), *Bufo americanus* (toad), and *Hyla versicolor* (gray tree frogs). Terrestrial experiments: *Rana sylvatica* (wood frog), *Bufo woodhousii fowleri* (Fowler's toad), and *Hyla versicolor* (gray tree frogs)

[48] Quoted from article

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Age of test organisms at study initiation:	Early stage appr. Gosner 25
Source:	Collected as newly oviposited eggs and emerged metamorphs, respectively from natural ponds in the vicinity of University of Pittsburgh. The gray tree frogs metamorphs for terrestrial experiments were taken from mesocosms.
Inoculation of mesocosm:	Sand/soil was added where applicable. 21 days prior to glyphosate application, 300 g of deciduous leaves (*Quercus* spp.), 25 g of rabbit chow and pond water containing algae and zooplankton were added. 2 days prior to glyphosate application, test organisms as tadpoles were added.
Handling prior to experiment:	20 tadpoles per species were set aside and assessed for 24-h survival due to handling.

4. Test system:

Study type:	Aquatic: mesocosm Terrestrial: laboratory
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	Aquatic: totally about 6 weeks with 21 days exposure Terrestrial: 24 hours.
Test conditions:	Aquatic: Experimental units were 1200-L cattle watering tanks filled with 1000 L of well water. Each tank was treated with no soil, sand or loamy soil. After inoculation of mesocosm and addition of test species, ponds were applied with test item or water (control) two day later. Survival was recorded 21 days later at termination of experiments. Terrestrial: Post-metamorphic animals were placed in 10-L plastic tubs that were lined with damp water towels. Subsequently, replicates were treated with glyphosate or water (control) and survival was recorded 24 h later.
Treatments:	Aquatic: total of 6 treatments including 3 soil treatments (i.e. no soil, 19 L sand, 19 L loam soil) crossed with 1 glyphosate and 1 control treatment. Terrestrial: 1 glyphosate and 1 control treatment.
Test concentrations:	Aquatic: 3.8 mg a.s./L (corresponding to 1.6 mL a.s./m^2) Terrestrial: 1.6 mL a.s./m^2
Replicates per treatment:	Aquatic: 5 Terrestrial: 4
Organisms per replicate:	Aquatic: 20 tadpoles of each of the 3 species in every mesocosm. Terrestrial: 7 juvenile frogs/toads per experimental unit separated by species.
Feeding during experiments:	No additional feeding
Parameters measured:	Aquatic: survival after 21 days exposure Terrestrial: survival after 24 hours.
Verification of test concentrations:	None analytics for aquatic experiments and none verification or

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calibration for precise application in terrestrial test system.

Validity criteria: None

5. Environmental conditions:

Test medium: Aquatic: well water while the used loam soil for the respective soil treatment was found to have the following composition: 29.2% sand, 21.4% clay and 49.4% silt).

Terrestrial:

Temperature: Not stated

Photoperiod/Light intensity: Natural light (May – June in Pittsburgh, USA)

pH: 8

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions.**

Comment:

- Source of used sand and soil is only vaguely described without any information about soil quality, contents, texture or chemical characteristics.
- The loam soil was taken from a field in the vicinity of the university but not tested for pesticide content. While it is stated that no pesticides were used on the field for several years the pesticide content in the solid remains unknown.
- The initial amount of algae and nutrition in the mesocosm ponds is not clearly given even though this is crucial for the survival of the test organisms and decides about the food availability throughout the experiment.
- Gray tree frogs showed 15% mortality in the 24-h assessment due to handling in mesocosm experiment indicating a high natural variability in survival of this species or high sensitivity to disturbances in aquatic experiments.
- Gray tree frogs were taken from mesocosms for terrestrial experiments without identifying the exact mesocosm they emerged from (treated or untreated, with or without soil).
- The concentrations reported to have resulted in toxicity, though never confirmed by measurement, was 3.8 mg GlyIPA/L. However, calculation to determine this concentrations did not take into account that the label's percentage of active ingredient is based on weight (not volume), and the density of Roundup branded product formulations is greater than 1 (1 mL of product equals more than 1 gram of product). "Roundup" applied to the cattle tanks was measured in milli litres, and then incorrectly assumed to contain an equivalent weight in grams. Correcting for density, nominal tested concentrations become 1.37 mg GlyIPA/L and 4.17 mg GlyIPA/L or 1.01 mg glyphosate a.e./L and 3.09 mg glyphosate a.e./L.

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- There is not enough information presented in the paper to determine what the actual application rate was that was sprayed over the juvenile animals to simulate a terrestrial exposure, or how the product was sprayed and whether any sort of sprayer calibration was attempted.

2. Relevance of study: **Relevant with restrictions**

Comment: Even though experimental approaches are reported validated in a few studies before, it lacks crucial information for a reliable assessment of most of the results.

Important to assessing the relevance of the terrestrial exposure part of the study, the exposure conditions were very artificial, plastic tubs lined with wet paper towels. Furthermore, the Roundup branded lawn and garden formulation used for this test is not used for agricultural or forestry applications. This lawn and garden formulation contains other components that could be toxic to frogs that are not found in Roundup branded agricultural or forestry products. **It is inappropriate to use this formulation to assess toxicity to terrestrial amphibians.**

This study tested concentrations of glyphosate that greatly exceeded predicted field exposure concentrations and PECsw from FOCUS step 1 of 0.101 mg/L and the duration of exposure. Tested rates represent direct over spray. In aquatic experiments, the used loam soil was not sufficiently analysed. Availability of food to tadpoles is unclear so that animals could also have died of starvation, particularly following reduction of algae due to herbicide use in ponds.

Gray tree frogs were proved to be an unsuitable test system in this study revealing a high natural variability of survival already at initialization of mesocosm experiment, and in terrestrial experiment it is unclear from which mesocosm individuals were taken.

The mesocosm studies with loam soil and gray tree frogs are judged to be not relevant and all others can only be considered with the limitation that mortality is attributed not only to direct glyphosate effect but also to indirect effects of reducing the available food.

Terrestrial trials on gray tree frogs are deemed to be not relevant due to lack of information about previous exposure.

All results besides tests with gray tree frogs and loam soil might be relevant considering that the test item corresponds with test item in evaluation, which is not given (formulation for home and garden use, containing POEA).

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

Author(s)	Year	Study title
Relyea, R.A., Schoeppner, N.M., Hoverman, J.T.	2005	Pesticides and amphibians: the importance of community context. Ecological Applications. Volume: 15 Issue: 4 Pages: 1125-1134 DOI: 10.1890/04-0559 ISSN: 1051-0761 (print)

Abstract[49]

The widespread application of pesticides has attracted the attention of ecologists as we struggle to understand the impacts of these chemicals on natural communities. While we have a large number of laboratory-based, single-species studies of pesticides, such studies can only examine direct effects. However, in natural communities, species can experience both direct and indirect effects. We conducted an outdoor mesocosm experiment on aquatic communities containing three tadpole species (*Hyla versicolor*, *Bufo americanus*, and *Rana pipiens*), zooplankton, and algae. We then manipulated a factorial combination of predators (no predators; newts, *Notophthalmus viridescens*; and larval beetles, *Dytiscus* sp.) and pesticides (no pesticides, the insecticide malathion, and the herbicide Roundup). We found that Roundup (1.3 mg of active ingredient/L) had substantial direct negative effects on the tadpoles, reducing total tadpole survival and biomass by 40%. However, Roundup had no indirect effects on the amphibian community via predator survival or algal abundance. Malathion (0.3 mg/L) had few direct effects on the tadpoles. Malathion caused no indirect effects with one of the predators (red-spotted newts) but caused substantial positive effects on amphibians (a five-fold increase in total tadpole survival and biomass) due to the sensitivity of the predatory beetles to the insecticide. Thus, while high concentrations of malathion can directly kill larval anurans, more ecologically relevant concentrations can have large positive effects in mesocosms by removing predatory insects. These results make it clear that pesticides can have both direct and indirect effects in natural communities and that these effects critically depend upon the composition of the community.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup ® (produced by Monsanto) but formulation details unknown
Active substance(s):	Glyphosate
Adjuvant / Surfactant:	polyethoxylated tallowamine (POEA) stated in paper but unconfirmed
Description:	Likely a lawn and garden formulation and composition unknown.
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	13 mg a. s./L, confirmed by the Mississippi State Laboratory (Mississippi State, Mississippi, USA) using high-pressure liquid chromatography.
Stock solution:	-

2. Vehicle and/or positive control: none

[49] Quoted from article

3. Test organism:

Species: Divers mesocosm community, especially tadpoles (American toads *(Bufo americanus),* leopard frogs *(Rana pipiens), and* gray tree frogs *(Hyla versicolor)*), newts (*Notophthalmus viridescens*), larval beetles (*Dytiscus* sp.) and algae

Age of test organisms at study initiation: Tadpoles were in their early development (initial mass was 55 ± 6 mg for leopard frogs, 19 ± 2 mg for toads, and 9 ± 1 mg for gray tree frogs [means 6 SE]).
Newt: 3.3 ± 0.1 g
Beetles: 0.23 ± 0.01 g and 0.10 ± 0.01 g

Source: Ponds in the vicinity of the University of Pittsburgh's Pymatuning Laboratory of Ecology in north-western Pennsylvania (USA)

Holding conditions prior to test: Eggs from amphibians were collected 1-2 month before application and were hatched in wading pools, hatchlings were fed with rabbit chow ad libitum

Acclimatisation: tadpoles had an acclimation period of two days, the first 6 h the predators were caged

4. Test system:

Study type: Lentic mesocosm study

Guideline: None

GLP: Not stated

Guideline deviations: -

Duration of study: 21st – 23rd April tanks are filled with water
22nd May – 12th June 2003

Test conditions: 1200 L cattle watering tanks filled with 1000 L well water

Application: Overspray of 10 mL (= 10 g) Roundup ® per tank, corresponding with 13 % a. s.

Application devices: Not stated

Application verification: Not stated

Treatments: 9

Test concentrations: 1.3 mg a.s./L (believed to be glyphosate IPA salt at this test concentration)

Replicates per treatment: 5

Organisms per replicate: 20 individuals of each species to each tank (= 8 tadpoles per species per square meter)

Feeding during experiments: -

Parameters measured: Survival, abundance, phytoplankton (chlorophyll a) abundance, biomass, growth of predatory newts and predaceous diving beetles

Analytical determination of test concentrations: None

Validity criteria: None

5. Environmental conditions:

Test medium: Natural matured well water inoculated with pond water

Temperature: Not stated

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Photoperiod: Not stated
Light intensity Not stated
pH: Not stated
Oxygen saturation Not stated
Conductivity: Not stated
Hardness: Not stated
Alkalinity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- The glyphosate formulation used in testing is referred to by the authors as “Roundup®”. ‘Roundup®’ represents a whole range of different formulations containing glyphosate. It is unclear which formulation was tested.
- As algae represent primary nutrients for aquatic ecosystems and an herbicide is applied, effects on algae need to be reported in detail (biomass or chlorophyll a).
- There is no analytical confirmation of test concentrations reported, but the results based only on nominal concentrations
- The reported concentrations, though never confirmed by measurement, were 1.3 mg GlyIPA/L. However, the author’s calculation to determine these concentrations did not take into account that the label’s percentage of active ingredient is based on weight (not volume), and the density of Roundup branded product formulations is greater than 1 (1 mL of product equals more than 1 gram of product). “Roundup” applied to the cattle tanks was measured in milli litres, and then incorrectly assumed to contain an equivalent weight in grams. Correcting for density, nominal tested concentrations become 1.37 mg GlyIPA/L or 1.01 mg glyphosate a.e./L.
- Although effect concentrations are discussed, no dose response curves and effect concentrations are presented
- Duration of the mesocosm study is very short, therefore no recovery of community could be investigated
- In mesocosm studies, a high natural (biological) variability is expected, but nothing is reported here. Statistics applied for evaluation of species richness are not appropriate; an evaluation using standardised tools such as the Shannon's diversity H′ or the Simpsons Diversity Index is missing.

2. Relevance of study: **Not relevant**

Comment: The study provides some relevant data while interpretation of results is difficult due to some weakness in experimental

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procedure, i.e. lack of analytics, insufficient identification of test items and test organisms.

This study tested what is believed to be a concentrated lawn and garden formulation that is not similar to the lead formulation in the glyphosate re-evaluation.

This study tested concentrations of glyphosate (1.3 mg a.i./L) that greatly exceeded predicted field exposure concentrations and PECsw from FOCUS step 1 of 0.101 mg/L and the duration of exposure. Tested rates represent levels only produced from a direct overspray.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment**

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Author(s)	Year	Study title
Relyea, R., Hoverman, J.	2006	Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology letters. Volume: 9 Pages: 1157-1171 DOI: 10.1111/j.1461-0248.2006.00966.x ISSN: 1461-023X (print), 1461-0248(online)

Abstract[50]

The field of ecotoxicology is experiencing a surge in attention among ecologists as we gain a deeper appreciation for how contaminants can impact natural ecosystems. This interest is particularly strong in aquatic systems where many non-target organisms experience pesticides. In this article, we assess how pesticides affect freshwater systems by applying the conceptual framework of density- and trait-mediated indirect effects from the field of basic ecology. We demonstrate the utility of this framework for understanding the conditions under which pesticides affect species interactions, communities and ecosystems. Through the integration of laboratory toxicity tests and this ecological framework, ecotoxicologists should be better able to identify the mechanisms through which pesticides affect communities and ecosystems. We also identify several areas of research that are in critical need of empirical attention including synergistic effects between pesticides and natural stressors, the importance of pesticides on community assembly via habitat preferences and oviposition effects, the timing and frequency of pesticide applications, pesticide effects on population dynamics, the evolution of pesticide resistance in non-target organisms and ecosystem recovery. With this knowledge, one can improve upon management decisions and help protect nontarget species that are of conservation concern.

KLIMISCH EVALUATION

1. Reliability of study: **Not assignable.**

Comment: This article is a literature review over 92 studies published in the years between 1977 and 2006. It summarizes the impact of pesticides in general on aquatic systems without giving details about material and methods allowing no evaluation of the reliability of the results.

2. Relevance of study: **Not relevant**

Comment: This is a literature review which may identify relevant concerns in ecology and the use of pesticides.

3. Klimisch code: **Klimisch rating of 4.**

[50] Quoted from article

Author(s)	Year	Study title
Relyea, R.A.	2012	New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. Ecological Applications. Volume: 22 Issue: 2 Pages: 634-647 DOI: 10.1890/11-0189.1 ISSN: 1051-0761

Abstract[51]

The use of pesticides is important for growing crops and protecting human health by reducing the prevalence of targeted pest species. However, less attention is given to the potential unintended effects on nontarget species, including taxonomic groups that are of current conservation concern. One issue raised in recent years is the potential for pesticides to become more lethal in the presence of predatory cues, a phenomenon observed thus far only in the laboratory. A second issue is whether pesticides can induce unintended trait changes in nontarget species, particularly trait changes that might mimic adaptive responses to natural environmental stressors. Using outdoor mesocosms, I created simple wetland communities containing leaf litter, algae, zooplankton, and three species of tadpoles (wood frogs [*Rana sylvatica* or *Lithobates sylvaticus*], leopard frogs [*R. pipiens* or *L. pipiens*]. and American toads [*Bufo americanus* or *Anaxyrus americanus*]. I exposed the communities to a factorial combination of environmentally relevant herbicide concentrations (0, 1, 2, or 3 mg acid equivalents [a.e.]/L of Roundup Original MAX) crossed with three predator-cue treatments (no predators, adult newts [*Notophthalmus viridescens*) or larval dragonflies [*Anax junius*]). Without predator cues, mortality rates from Roundup were consistent with past studies. Combined with cues from the most risky predator (i.e., dragonflies). Roundup became less lethal (in direct contrast to past laboratory studies). This reduction in mortality was likely caused by the herbicide stratifying in the water column and predator cues scaring the tadpoles down to the benthos where herbicide concentrations were lower. Even more striking was the discovery that Roundup induced morphological changes in the tadpoles. In wood frog and leopard frog tadpoles, Roundup induced relatively deeper tails in the same direction and of the same magnitude as the adaptive changes induced by dragonfly cues. To my knowledge, this is the first study to show that a pesticide can induce morphological changes in a vertebrate. Moreover, the data suggest that the herbicide might be activating the tadpoles' developmental pathways used for antipredator responses. Collectively, these discoveries suggest that the world's most widely applied herbicide may have much further-reaching effects on nontarget species than previous considered.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Original MAX®
Active substance(s):	Glyphosate
Adjuvant / Surfactant:	Undisclosed surfactant
Description:	General information about glyphosate background is described in article.
Source of test substance:	Not stated (manufactured by Monsanto Corporation, St. Louis, Missouri, USA)
Lot/Batch #:	Not stated

[51] Quoted from article

Purity: 540 mg a.e./L

Stock solution: Herbicide was dissolved in 300 mL container of water and subsequently drizzled across mesocosm surface at treatment.

2. Vehicle and/or positive control: Not applicable

3. Test organism:

Species: *Rana sylvatica* (wood frog)
Rana pipiens (northern leopard frog)
Bufo americanus (American toad)

Age of test organisms at study initiation: Early stage appr. Gosner 25

Source: Collected as newly oviposited eggs from natural ponds in the vicinity of University of Pittsburgh.

Handling of species prior to experiment: Eggs hatched in aged well water and hatchlings were fed rabbit chow ad libitum. Tadpoles for experiments were taken from 10-15 different clutches per test species.

Inoculation of mesocosm: 20 days prior to experiment, 200 g of deciduous leaves (*Quercus* spp.), and 15 g of rabbit chow and pond water containing zooplankton, phytoplankton and periphyton were added. Mesocosms were covered with 65% shade cloth lids to prevent other organisms from ovipositing. Tadpoles of each species were added to every tank according to treatments.

4. Test system:

Study type: Mesocosm

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 21 days

Test conditions: Experimental units were 757-L cattle watering tanks filled with 570 L of well water. At each concentration and untreated control, two predator treatments were applied, i.e. predator present or absent. Predator adult red-spotted newt (*Notophthalmus viridescens*) or larval dragonflies (*Anax junius*) were added in cages so that emitted chemical cues can diffuse through water and induce prey stress (method validated by previously published studies). Predator cages were screened plastic cups with 1 adult newt for predator presence and empty for predator absence treatment.

Treatments: Total of 12 treatments including 3 different concentrations of single glyphosate applications and 1 untreated control crossed with 3 predator treatments (no predator, caged adult newt, or caged larval dragonflies).

Test concentrations: 1, 2 and 3 mg a.e./L glyphosate (a.e. = acid equivalent).

Replicates per treatment: 4

Organisms per replicate: 90 tadpoles in total (30 tadpoles of each of the 3 test species)

Feeding during experiments: No additional feeding

Parameters measured: Survival and individual mean mass at termination of experiment

Verification of test concentrations: Actual concentration of glyphosate was analysed for medium

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level of the water column 1 hour after glyphosate application by using HPLC.

Validity criteria: None

5. Environmental conditions:

Test medium: Well water

Temperature: 11.7 – 11.9°C (measurements on day 9)

Photoperiod/Light intensity: Natural light with 65% shade cloth covering mesocosm tanks (May - June in Pittsburgh, USA)

pH: 7.8-8.0 (measurements on day 9)

Dissolved oxygen: 10 - 20 mg/L on day 9

Periphyton: Biomass increased over time across all treatments while significantly more periphyton was found in 3 mg a.e./L treatment..

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Tanks were shaded by cloth lid with rather low impact on mesocosm as periphyton was still observed to grow during experimental time. Question arises with respect to significantly more periphyton in the highest concentration of herbicides.
- Actual concentrations were analysed to deviate within 13% of nominal values and authors referred to nominal concentrations in the article for simplifications. Thus, it remains unclear whether probit analyses are based on nominal or actual concentrations.
- Dissolved oxygen varied greatly among mesocosm ponds requiring analysis of treatment effects on dissolved oxygen. Dissolved oxygen was found to depend on herbicide treatment.

2. Relevance of study: **Not relevant**

Comment: The current study uses a highly artificial test system and tests concentrations that are not relevant to actual environmental concentrations resulting from the proper application of Roundup Original MAX or other glyphosate-surfactant herbicide formulations. The tested concentrations can only be achieved by application of Roundup Original MAX at field rates of 1.12 to 4.6 kg glyphosate acid equivalent (a.e.)/ha sprayed directly over very shallow bodies of water. MON 52276 is not labelled for direct application over aquatic environments.

Small differences in body measurements reported by Relyea are of questionable biological relevance and occur in nature as a result of various stressors, including predation. Thus, it is difficult to extrapolate these findings to a natural environment where predation and other stressors are both common and highly variable. This work fails to identify any new risk to amphibians and raises no new concerns regarding the environmental safety of Roundup Original MAX or other

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glyphosate-surfactant herbicide products.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

Further considerations:

1. The study by Relyea identifies no new risk to amphibians, and the tested concentrations greatly exceeded environmentally relevant levels.

2. These concentrations could only be obtained by direct application of Roundup Original MAX to 15 cm of water at application rates of 1.12 to 4.6 kg glyphosate acid equivalent (a.e.)/ha. Roundup Original Max is not approved for direct application to water. The author acknowledges this very important point in this paper.

3. An operational monitoring study by Thompson *et al*. 2004 demonstrated that exposures typically occurring in forest wetlands are insufficient to induce significant acute mortality in native amphibian larvae. Solomon et al. (2009) demonstrated that under conditions of exposure in the field, interception by foliage and adsorption to soils and sediments reduce exposures still further; and risks, even to a direct overspray, are small to negligible.

4. Field exposures to water bodies outside the cultivated field will be from spray drift. These exposures from drift will be a small fraction of the concentrations tested by Relyea and would not result in the reported effects to tadpoles.

5. LC_{50} values (the concentration required to cause 50% mortality) for the three species of tadpoles were comparable across species and treatments (with and without predators) narrowly ranging from about 2.4 to 3.0 mg glyphosate a.e./L. These differences in LC_{50} values, as shown in the paper, are not significantly different in all treatments.

6. This paper does not provide sound evidence of morphological changes as a result of exposure to a glyphosate-based formulation. Measured differences in morphological measurements were exceedingly small and are presented in a misleading manner (Figure 3), using limited axes that artificially magnify small differences across treatments. The method used for morphological measurement is not described, and is critical because very small differences are claimed to be significant. These differences are as small as 0.01 cm, less than 5% among treatments, and are not considered to be biologically meaningful. Typically, differences less than 0.1 cm are not considered significant and are less than the resolution of the measurement method, where rounding is done to the nearest millimeter.

7. Dissipation and degradation of glyphosate and the surfactant in natural systems will rapidly occur. This will significantly decrease the duration of exposure, perhaps to no more than 1 or 2 days following an application (Wang et al., 2005). Contrary to what is reported in the paper, the presence of sediment in exposure systems will reduce toxicity to tadpoles (Bernal et al. 2009).

8. The observed worst-case concentrations cited in this paper (from Giesy et al. 2000 and Edwards et al. 1980) occurred at application rates that exceed the maximum labelled rates for in-crop use of glyphosate in Roundup Ready crops by up to five times. When the maximum observed concentrations reported in Giesy et al. (2000) and Edwards et al. (1980) are proportionally adjusted to predict a concentration at the maximum in-crop application rate, the estimated concentrations are 1.0 mg a.e./L or less. At a concentration of 1.0 mg a.e./L no morphological differences were observed compared to untreated controls. In the third reference cited (Thompson et al. 2004), Dr. Relyea acknowledges that the high concentration cited is not representative of the wetlands treated in Thompson et al. (High concentration is almost six times the mean of all

similarly treated water bodies in the study)

9. Considering the rates of the glyphosate formulation tested, the depth of the aquatic system used in this study is, at a minimum, nearly three times deeper than a natural water body would be that had agriculturally relevant rates of the glyphosate formulation applied by a direct overspray (an application in violation of the EPA-approved label). The physical and biological properties of a 16-inch deep water body without sediment are expected to be much different than the properties of a 6-inch deep natural water body containing sediment. The ecological relevance of the results of this study are, therefore, open to question.

References:

Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos®) and Cosmo-Flux® to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity, J. Toxicol. Environ. Health A 72:966–973.

Edwards WM, Triplett GB, Kramer RM. 1980. A watershed study of glyphosate transport in runoff. J. Environ. Qual. 9:661-665.

Giesy JP, Dobson S, Solomon KR. 2000. Ecotoxicological risk assessment for Roundup herbicide. Rev, Envirion. Contam. Toxicol, 167:35-120.

Thompson D, Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian lavae in forest wetlands. Environ. Toxicol. Chem. 23(4): 843-849.

Solomon KR, Marshall EJ, Carrasquilla G, 2009. Human health and environmental risks from the use of glyphosate formulations to control the production of coca in Colombia: overview and conclusions. J Toxicol Environ Health A. 72(15-16):914-920.

Wang N, Besser JM, Buckler DR, Honegger JL, Ingersoll CG, Johnson BT, Kurtzweil ML, MacGregor J, McKee MJ. 2005. Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms. Chemosphere 59: 545–551.

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Author(s)	Year	Study title
Williams, B.K., Semlitsch, R.D.	2010	Larval Responses of Three Midwestern Anurans to Chronic, Low-Dose Exposures of Four Herbicides Archives of Environmental Contamination and Toxicology Volume: 58 Number: - Pages: 819-827 URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19768486 DOI: 10.1007/s00244-009-9390-z ISSN: 1432-0703 (Electronic)

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Abstract[52]

Low levels of agricultural herbicides often contaminate surface water and might persist throughout the growing season, potentially acting as stressors on aquatic organisms. Although low-dose, chronic exposures to agrochemicals are likely common for many nontarget organisms, studies addressing these effects using end-use herbicide formulations are rare. We exposed three common species of tadpoles to conservative levels of atrazine, S-metolachlor, and glyphosate end-use herbicide formulations throughout the larval period to test for survival differences or life-history trait alterations. Exposure to the glyphosate product Roundup WeatherMax® at 572 ppb glyphosate acid equivalents (a.e.) resulted in 80% mortality of western chorus frog tadpoles, likely as a result of a unique surfactant formulation. Exposure to WeatherMax® or Roundup Original Max® at 572 ppb a.e. also lengthened the larval period for American toads. Chronic atrazine and S-metolachlor exposures induced no significant negative effects on survival, mass at metamorphosis, or larval period length at the levels tested. These results highlight the importance of explicitly tying chronic tests to the natural environment and considering contributions of surfactant/adjuvant components to end-use formulation toxicities, even between very similar products.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup Original Max ® Roundup Weather Max®
Active substance(s):	Glyphosate K-salt
Surfactant:	Not stated
Description:	none
Source of test substance:	Producer: Monsanto Company, St. Louis, MO
Lot/Batch #:	Not stated
Purity:	Roundup Original Max ®: 48.7% glyphosate K-salt Roundup Weather Max®: 48.8% glyphosate K-salt
Stock solution:	Stock solutions were prepared by mixing end-use products (amounts not stated) with distilled water. At each renewal 0.1 mL of stock solution was pipetted into each jar.

2. Vehicle and/or positive control: none

[52] Quoted from article

3. Test organism:

Species:	*Bufo americanus* *Pseudacris triseriata* *Hyla versicolor*
Age of test organisms at study initiation:	Tadpoles, Gosner stage 25
Source:	Egg strings (*Bufo americanus*) and masses (*Pseudacris triseriata*) collected from two wetlands in Union ridge Conservation Area (MO, USA). Adult *Hyla versicolor* were collected at pond at Thomas S. Basket Wildlife Research and Education Area (MO, USA) and allowed to oviposit in the laboratory.
Holding conditions prior to test:	Eggs were maintained at 25 °C through hatching until Gosner Stage 25 (begin of independent feeding).
Acclimatisation:	Not stated

4. Test system:

Study type:	Static renewal (3 days)
Guideline:	Not applicable
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	Until Gosner stage 42
Test conditions:	Tadpoles were raised individually in 1 L glass jars.
Replicates per concentration:	10
Organisms per replicate:	1
Feeding:	4.5 mg/tadpole after each water change and increasing periodically as animals grew. *Bufo americanus* and *Pseudacris triseriata*: 50/50 mixture of ground Tetramin® fish flakes and ground commercial rabbit chow *Hyla versicolor*: 100% ground fish flakes
Parameters measured:	Survival, mass at metamorphosis (Gosner stage 42), length of larval period.
	Survival was evaluated by logistic regression; mass at metamorphosis and time to metamorphosis were evaluated using univariate ANOVA and Tukey-Kramer as post-hoc test. No EC/LC_{50} values were determined.
Test concentrations:	0.6 and 700 µg a.s./L, equivalent to 0.5 and 572 µg a.e./L
Analytical determination of test concentrations:	Not stated
Validity criteria:	Not applicable

5. Environmental conditions:

Test medium:	UV disinfected, carbon filtered water
Temperature:	25 °C
Photoperiod:	16 h
Light intensity	Not stated
pH:	7.8
Oxygen saturation	Not stated

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Conductivity: ~688 µS/cm
Hardness: ~232 mg $CaCO_3$/L
Alkalinity: ~270 mg $CaCO_3$/L

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (omissions in experimental descriptions)

Comment
- No analytical verification of test substance
- Formulation of unknown origin/content of adjuvants or surfactants.
- Unclear how exposure concentrations were derived, i.e. formulation translates to a.s./a.e..
- Spacing between concentrations extremely high.
- Wild tadpoles, no history of previous exposure/toxic stress stated/evaluated

2. Relevance of study: **Not relevant**

Amphibian risk assessment not an EU requirement and the study was not performed with the lead formulation, MON 52276 under evaluation

3. Klimisch code: This results in a **Klimisch rating of 3**

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Effects on Aquatic Invertebrates

From the data presented in the core dossier it becomes clear that acute and chronic toxicity of glyphosate to aquatic invertebrates is low and comparable to fish. As discussed in the section that provides an overview for fish toxicity, toxicity of the formulations to aquatic invertebrates can be driven by the surfactant and the site of action for this nonspecific toxicity is the gill; and only occurs at concentrations that greatly exceed environmentally realistic field concentrations.
Below is the review for approximately one dozen studies that evaluated potential effects on aquatic invertebrates.

Author(s)	Year	Study title
Achiorno, C.L., Villalobos, C., Ferrari, L.	2008	Toxicity of the herbicide glyphosate to *Chordodes nobilii* (Gordiida, Nematomorpha) Chemosphere Volume: 71 Issue: 10 Pages: 1816-1822 URL: http://www.sciencedirect.com/science/article/pii/S004565350800163X DOI: 10.1016/j.chemosphere.2008.02.001 ISSN: 0045-6535

Abstract[53]

Nematomorpha (horsehair worms) is a poorly known group of worm-like animals similar to nematodes. Adults are free-living and reproduction takes place in freshwater environments, where preparasitic larvae undergo development. All species have a parasitic juvenil stage and infection may result in the host's death, insects being the most frequent host. Most of the life cycle occurs in freshwater environments, which are often contaminated by different pollutants. Based on the lack of information on the toxicity of herbicides to horsehair worms, the objective of this study is to evaluate the effect of different concentrations of glyphosate (technical grade and formulated product) on *Chordodes nobilii* (Gordiida, Nematomorpha). Bioassays were performed with embryos and larvae (preparasitic stages), and adults (postparasitic stage). Test organisms were exposed for a short period of time to concentrations ranging between 0.1 and 8 mg a.e. l^{-1} of glyphosate (technical and formulated). Although embryo development was not inhibited, there was a significant decrease in the infective capacity of larvae derived from eggs that had been exposed to 0.1 mg/l. Similar results were obtained for directly exposed larvae. No differences in toxicity were detected between the active ingredient and formulated product. Adult exposed for 96 h to 1.76 mg l^{-1} formulated Gly shown a mortality of 50%. Results indicate that *C. nobilii* is affected at glyphosate concentrations lower than those expected to be found in freshwater environments and those specified in the legislation.

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate technical 'A formulation like Roundup'
Active substance(s):	Not stated
Surfactant:	Not stated
Description:	Not stated, not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
Source of test substance:	Servicio Nacional de Sanidad y Calidad Agroalimentaria de

[53] Quoted from article

	Argentina (SENASA)
Lot/Batch #:	Not stated
Purity:	Glyphosate technical: 95% (w/v) Formulation: 35.2% formulated glyphosate (w/v) Purity verified by SIPAC technique
Stock solution:	500 mg a.e./L glyphosate in bi-distilled water.
2. Vehicle and/or positive control:	Second to third instar larvae of *Aedes aegyptii* as vector for *C. nobilii* larvae.
3. Test organism:	
Species:	*Chordodes nobilii*
Age of test organisms at study initiation:	Embryos mainly in blastula stage derived from egg strings laid by a field collected female larvae and adults.
Source:	Adults collected from streams in the locality of Sierra de La Ventana, Buenos Aires, Argentina
Holding conditions prior to test:	Individuals from each stream were kept in separate containers with aerated dechlorinated tap water at a room temperature of 23 ± 1 °C. After mating, females were held individually in containers for oviposition. For the embryo test egg strings were cut into segments of about 3 mm length, each containing approx. 4000 eggs.
4. Test system:	
Study type:	Semi-static for embryos and larvae, static for adults
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of exposure:	Embryo test: 96 h Larvae bioassay: 48 h
Test conditions:	Tests were conducted in rearing chamber in full darkness to avoid product degradation. Embryo test: egg segments were placed in 1.5 mL containers filled with the test item solution resulting in an egg density of approx. 2500-3000 eggs/mL. Medium was renewed partially daily and after exposure replaced by control medium. For exposure of *Aedes aegyptii* larvae to *C.nobilii*, 30 second to third instar larvae of *A. aegyptii* were exposed for 72 h to larvae of *C. nobilii* in containers filled with 12 mL well water and fed with wheat germ. Larvae bioassay: egg segments were placed in 50 mL plastic containers until free-living stage (FL) was reached. FL stages were then exposed for 48 h to test item concentrations. Medium was renewed partially after 24 h and after exposure replaced by control medium. Then, FL were exposed for 72 h to larvae of *A. aegyptii* in control medium. Adult bioassay: adult males were exposed in 500 mL of assay medium.
Replicates per concentration:	3 (embryo and larvae) 4 (adults)
Organisms per replicate:	Embryo and larvae test: not stated

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Adult test: 5

Feeding: None/not applicable

Parameters measured: Embryo and larvae test:
proportion of free-living larvae, ready-to-hatch-larvae within egg capsules and non-viable embryos until amount of free-living larvae was >50% (28 to 38 days after start of assay). Infection Index Mean Abundance (IIMA) following exposure for 72 h of larvae to larvae of *Aedes aegyptii*.
Adult bioassay:
Mortality was registered at 96 h post exposure.Significant differences were determined by one-way ANOVA followed by Tukey's test as post-hoc. For the adult bioassay, Student's t-test was used for comparison between control and treated group.

Test concentrations: Embryo and larvae bioassays: Glyphosate (technical): 0, 0.1, 0.5, 1, 2, 4 and 8 mg a.e./L
Formulated glyphosate: 0, 0.07, 0.4, 0.8, 1.5, 3 and 6 mg a.e./L
Adults bioassays: 1.76 mg a.e./L formulated glyphosate

Analytical determination of test concentrations: Analytical concentrations of stock solutions were determined by ionic chromatography with chromatographic columns AG4-AS4, analytical values differed ± 10% of nominal values.

5. Environmental conditions:

Test medium: Reconstituted hard water:
pH 7.6 – 8.0
Hardness: 160 – 180 mg/L as CO_3Ca
120 mg/L $MgSO_4$
192 mg/L $NaHCO_3$
8 mg/L KCl
120 mg/L $CaSO_4 \cdot 2H_2O$

Temperature: 23 ± 1 °C

Photoperiod: Full darkness

pH: 7.04 – 7.47 (test medium at start)
final pH increased less that 4% in all cases

Oxygen saturation Not stated

Conductivity: Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (study carried out according to a method which is not acceptable, documentation not convincing for an expert judgement).

Comment:

- Major omissions in reporting of experimental details; for embryo and larval bioassay, no oxygen concentration stated, it is not stated, how many specimens were exposed to *A. aegyptii*. Not clear for how long adults were exposed, it is just reported that mortality was registered 96 h post exposure.
- It is not reproducible what test substance was used as formulation.
- Unclear why only adult males were exposed, but no

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females.

2. Relevance of study: **Not relevant**

Comment: Due to substantial methodological flaws, study is not considered to be relevant.

3. Klimisch code: A clear description or specification of the test substance (referred to as glyphosate formulation like Roundup) is missing; no content of active substance is stated. This renders the study 'not reliable', resulting in a **Klimisch rating of 3.**

Further considerations:

In this study, *Chordodes nobilii* (Gordiida, Nematomorpha) embryos, larvae (preparasitic stages), or adults (postparasitic stage) were exposed for a short period of time to concentrations of glyphosate (technical and formulated) ranging between 0.1 and 8 mg a.e. /L. Although embryo development was not inhibited, the authors reported a significant decrease in the infective capacity of larvae derived from eggs that had been exposed to ≥0.1 mg/l. The authors reported similar results for directly exposed larvae. No differences in toxicity were detected between the active ingredient and formulated product. Adults exposed for 96 h to 1.76 mg a,e/L. of formulated glyphosate were reported to display a mortality of 50%.

General Comments:

Chordodes nobilii (Gordiida, Nematomorpha) is a not a well recognized test species. In fact, the authors report that "there is no information on the effects of different pollutants on the life cycle stages of gordiids". No validated testing methodology for such studies is therefore currently available for this species. While the test results may be interesting from an academic perspective, there are a number of methodological and ecological questions pertaining to this study that need to be addressed before the results could be considered useful for environmental risk assessment.

Specific comments:

1. Scientifically, exposure of larvae in isolation would not occur in the field. The host (insect larvae - in this case *Aedes aegyptii* larvae) would be exposed as well, so the test design does not reflect a relevant environmental exposure, and, therefore, the reported results are not directly relevant for environmental risk assessment.
2. As a nematomorph, the parasite-host interactions are often extremely complicated and poorly understood. It would be important to understand how these interactions react to a number of varying test substances and test conditions before drawing conclusions for any specific test substance. The authors have recently published another paper examining effects of three reference toxicants to embryonic and larval stages of *Chordodes nobilii* (Achiorno et al. 2010), but glyphosate was not included in comparative tests with these reference toxicants. The variability of results over time for test substances compared to reference toxicants has not been assessed.
3. With respect to quantifying parasitation success, there is no specific guideline for conducting such a study. Although there is a standard guideline (ISO 10872/FDIS) for nematodes, it is based on a full life cycle test using free-living bacterial feeding nematodes. Impacts on nematomorphs currently are not considered (most likely due to the complexity of parasite + host interactions).
4. The authors did not show the data with the adult bioassays. They mention it in the results but show no data and never really discuss it.
5. The infection assay is a fairly standard assay that is commonly used, but it is not designed to make projections of population survivability.
6. While the data appear to show decreases in infectivity it could be caused by a wide variety of factors and may not necessarily be due to "toxicity". There are many ways to interrupt the host-pathogen relationship that have nothing to do with "toxicity". Additionally, it has been demonstrated in plants

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that reducing initial infection can often lead to those successfully infecting being better pathogens and having higher fecundity.

7. The authors talk about the decline of viability in its ecological role but there is no data that indicates that there is a decline. It is possible that even with a reduced number of worms infecting, their ability to reproduce given less competition may be enhanced or at least balanced out. The paper makes claims well beyond the data provided.
8. The glyphosate formulation used in this study is not identified with sufficient detail to determine if this formulation could be similar to the lead formulation MON 52276.
9. The conclusions state "that this information can be used by government decision-makers when taking action to promote conservation of biodiversity in freshwater ecosystems". Given the questions over the species selection and the test design, further work is needed before the relevance of this study's endpoints to environmental risk assessment can be determined...

References (Review of Achiorno et al. 2008)

Achiorno CL, de Villalobos C, Ferrari L. (2008) Toxicity of the herbicide glyphosate to *Chordodes nobilii* (Gordiida, Nematomorpha). Chemosphere 71(10): 1816–1822. doi:10.1016/j.chemosphere.2008.02.001

Achiorno CL, de Villalobos C, Ferrari L. (2010) Validation test with embryonic and larval stages of *Chordodes nobilii* (Gordiida, Nematomorpha): Sensitivity to three reference toxicants. Chemosphere 81: 133–140. doi:10.1016/j.chemosphere.2010.06.076

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Author(s)	Year	Study title
Brausch, J.M., Cox, S., Smith, P.N.	2006	Pesticide usage on the Southern High Plains and acute toxicity of four chemicals to the fairy shrimp *Thamnocephalus platyurus* (Crustacea: Anostraca). The Texas Journal of Science Volume: 58 Issue: 4 Pages: 309-324 Publication not available online DOI: not applicable ISSN: 00404403

Abstract[54]

Extensive pesticide use on crops grown on the Southern High Plains (SHP) represents a considerable anthropogenic stressor to ephemeral aquatic ecosystems. These short-lived aquatic ecosystems, known in the southwest as playas, are epicentres of biodiversity on the High Plains. Cotton is the major agricultural crop grown on the SHP, accounting for over half of all cotton produced in Texas.

Currently there are 67 different chemicals used to control cotton pests, and when other crops such as grain sorghum are included the number approaches 100. *Thamnocephalus platyurus* is a fairy shrimp indigenous to the Southern High Plains that is also available commercially. In addition it is used as an invertebrate model for water quality and toxicity testing. Acute toxicity of four agricultural pesticides widely used on the SHP (Methyl Parathion 4E, Tempo® SC Ultra [active ingredient cyfluthrin], Roundup® [glyphosate], and Karmex® DF [diuron]) was determined using laboratory derived *T. platyurus*. Twenty-four hour old nauplii experienced mortality (48 hour LC_{50}) at concentrations ranging from 10.99 µg/L for Tempo® SC Ultra and 1.248 mg/L for Roundup®. These results suggest that the current pesticide application rates have the potential to endanger the native playa invertebrate *T. platyurus*.

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Super Concentrate®
Active substance(s):	Glyphosate acid
Surfactant:	Polyethoxylated tallowamine (POEA)
Description:	Not stated, not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested. No a.s. loading reported.
Source of test substance:	Monsanto Company, St. Louis, MO
Lot/Batch #:	Not stated
Purity:	Not stated
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	*Thamnocephalus platyurus*
Age of test organisms at study initiation:	nauplii used within 24 hours of hatching (i.e. <24 post-hatch)
Source:	Thamnotoxkit F, Creasel Ltd. Belgium

[54] Quoted from article

Holding conditions prior to test: Cysts (dormant eggs) were hatched in moderately hard synthetic fresh water acc. to EPA/600/4-90/027F

4. Test system:

Study type: Static

Guideline: None; study design comparable to ISO 14380:2011

GLP: No

Guideline deviations:
- 48 h instead of 24 h
- No analytics provided.

Duration of study: 48 h

Test conditions: Tests were conducted in 250 ml beakers filled with 175 ml of EPA synthetic fresh water. At 4, 8, 12, 24 and 48 hours after addition of the chemical, dead nauplii were counted.

Replicates per concentration: 3

Organisms per replicate: 20

Feeding: None

Parameters measured: Mortality at 4, 8, 12, 24 and 48 hours, water quality parameters three times for the highest test concentration.
Mortality data were evaluated using logit analysis (R ver. 2.0.1)

Test concentrations: 19.9, 199, 747, 1595, 4175, 19920 and 199200 µg Roundup®/L (nominal)

Analytical determination of test concentrations: Not measured

5. Environmental conditions:

Test medium: Synthetic fresh water: EPA/600/4-90/027F (EPA 1993):
60 mg/L $MgSO_4$
96 mg/L $NaHCO_3$
4 mg/L KCl
60 mg/L $CaSO_4 \cdot 2H_2O$

Temperature: 24.6 ± 0.2 °C

Photoperiod: 14 h light

Light intensity: 1.575 lux

pH: 7.53 (test medium at start)
5.05 ± 0.05 (highest test concentration)

Oxygen saturation: 8.27 ± 0.04

Conductivity: Not stated

Hardness: 66 mg $CaCO_3$/L

Alkalinity: 92 mg $CaCO_3$/L

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (study carried out according to a method which is not acceptable, documentation not convincing for an expert judgement).

Comment:
- Clear description of the test substance missing; not clear which formulation (e.g. concentrated lawn or

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garden, agricultural formulation) was tested.

- Specification of test substance missing (purity, contents of a.s.), content of a.s. not stated.
- Exposure period exceeds the recommended duration of 24 h; without feeding, neonates of *Thamnocephalus platyurus* will suffer starvation after 24 h which exhibits additional ecological stress.
- Due to its small size, the kit requires that the shrimps are tested in a small volume of water (1 mL), but test was conducted in 175 mL

2. Relevance of study: **Not relevant**

Comment: Due to substantial methodological flaws, study is not considered to be relevant.

3. Klimisch code: A clear description or specification of the test substance is missing; no content of active substance is stated. The manufacturer of the Thamnotoxkit F, which was used for the current study, recommends a maximum exposure duration of 24 hours, as neonates will not survive for more than 24 hours at good health without food supply. This renders the study 'not reliable', resulting in a **Klimisch rating of 3 and not adequate for risk assessment.**

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	Year	Study title
Brausch, J.M., Smith, P.N.	2007	Toxicity of Three Polyethoxylated Tallowamine Surfactant Formulations to Laboratory and Field Collected Fairy Shrimp, *Thamnocephalus platyurus*. Archives of Environmental Contamination and Toxicology Volume: 52 Issue: 2 Pages: 217-221 DOI: 10.1007/s00244-006-0151-y ISSN: 0090-4341 (print), 1432-0703 (online)

Abstract[55]

Polyethoxylated tallowamine (POEA) is a non-ionic surfactant used in herbicide formulations to increase the efficacy of active ingredients. POEA promotes penetration of herbicide active ingredients into plant cuticles, and in animal species is known to cause alterations in respiratory surfaces. POEA use has increased recently with the advent of ''Roundup-Ready'' crops; however, its potential effects on aquatic invertebrates are relatively unknown. The aquatic macroinvertebrate *Thamnocephalus platyurus* (Crustacea, Anostraca) was used to assess the acute toxicity of POEA. Three formulations of POEA consisting of a 5:1, 10:1, and 15:1 average oxide:tallowamine were used in this study. All POEA formulations were found to be extremely toxic to *T. platyurus* with 48-h LC50 concentrations as low as 2.01 lg/L for 15:1. POEA toxicity increased as the tallowamine chain length was reduced, whereas the oxide chain length appeared to only slightly increase toxicity. Based on these results, POEA has the potential to adversely affect aquatic organisms in areas in which it is used.

MATERIALS AND METHODS

1. Test material:

Test item:	Surfonic® T-5, T-10 and T-15 surfactant
Active substance(s):	Polyethoxylated tallowamine (POEA)
Adjuvant:	The test item, Surfonic®, itself is the adjuvant and generally used with "Roundup-Ready" (a.s. glyphosate) while in this study surfactant was applied purely.
Description:	POEA is characterized by the mass ration between the oxide and tallowamine portions of the molecule. Average oxide : tallowamine ratios were 5:1 (T-5), 10:1 (T-10) and 15:1 (T-15).
Source of test substance:	Huntsman International LLC (Salt Lake City, UT, USA)
Lot/Batch #:	Not stated
Purity:	98.6%, 99.8% and 99.4% pure for T-5, T-10 and T-15, respectively

2. Vehicle and/or positive control: None

3. Test organism:

Species:	*Thamnocephalus platyurus*
Age of test organisms at study initiation:	24 hours old
Source:	Cysts used in Thamnotoxkit F (Creasel Ltd. Belgium) and cysts from wild-caught fairy shrimps collected in grassland playa

[55] Quoted from article

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lakes and in cropland playa lakes on the Southern High Plains, Texas (USA).

Holding conditions prior to test: Wild type female shrimps were fed with yeast and trout chow (Cerophyll®) mixture and kept until they deposited their cysts which were dried at 35°C for one week and subsequently stored at 4°C until toxicity testing.
All nauplii for testing (wild-caught and Thamnotoxkit F) were hatched in synthetic freshwater according to USEPA (2002) with pH = 7.3, alkalinity 63 mg/L, hardness 94 mg/L both as $CaCO_3$) under 14:10 light/dark regime with light intensity of 1575 lux and at 25 ± 0.2 °C.

4. Test system:

Study type: Static

Guideline: None; study design comparable to ISO 14380:2011

GLP: No

Guideline deviations:

- 48 h instead of 24 h
- No analytics provided.

Duration of study: 48 h

Test conditions: Tests were conducted in 250 ml beakers filled with 175 ml of EPA synthetic fresh water. Dead nauplii were counted at 6, 12, 24 and 48 hours of exposure.

Treatments: 3 different POEA formulation (ie. T-5, T-10 and T-15) treatments and 3 untreated control with each of 3 strains of shrimps (1 laboratory, 2 wild-caught).

Test concentrations: 7 test concentrations in each treatment: 0.01, 0.1, 10, 100, 1,000 and 10,000 µg/L.

Replicates per concentration: 3

Organisms per replicate: 20

Feeding: None during testing.

Parameters measured: Mortality at 6, 12, 24 and 48 hours, water quality parameters were measured three times throughout test.
Mortality data were evaluated using logit analysis (R ver. 2.2.1).

Analytical determination of test concentrations: Not measured

5. Environmental conditions:

Test medium: Synthetic fresh water according to EPA-821-R-02-012 (USEPA 2002).

Temperature: 24.7 ± 0.1 °C

Photoperiod: 14 h light

Light intensity 1.575 lux

pH: 6.3 – 6.8

Oxygen saturation 8.00 – 8.38 mg/L ± 0.04

Conductivity: Not stated

Hardness: 94 mg $CaCO_3$/L

Alkalinity: 63 mg $CaCO_3$/L

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Even though well-documented the method is not validated.
- Deviations from comparable guideline is not acceptable
- Exposure period exceeds the recommended duration of 24 h; without feeding, neonates of *Thamnocephalus platyurus* will suffer starvation after 24 h which exhibits additional ecological stress.
- Due to its small size, the kit requires that the shrimps are tested in a small volume of water (1 mL), but test was conducted in 175 mL

2. Relevance of study: **Not relevant**

Comment: Duration and provision of food is crucial for toxicity testing on *Thamnocephalus platyurus*. An assessment of mortality at 48 h instead of 24 h exposure does not allow conclusions to the stressors' effects.
The manufacturer of the Thamnotoxkit F recommends a maximum exposure duration of 24 hours, as neonates will not survive for more than 24 hours at good health without food supply. This renders the study 'not reliable'.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

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Author(s)	Year	Study title
Brausch, J.M., Blake, B., Smith, P.N.	2007	Acute and Sub-Lethal Toxicity of Three POEA Surfactant Formulations to *Daphnia magna*. Bulletin of Environmental Contamination and Toxicology. Volume: 78 Issue: 6 Pages: 510-514 DOI: 10.1007/s00128-007-9091-0 ISSN: 0007-4861 (print), 1432-0800 (online)

Abstract[56]

Polyethoxylated tallowamine (POEA) is a nonionic surfactant used in many herbicide formulations to increase the ability of active ingredients to penetrate leaf cuticles. However, it has also been shown to disrupt respiratory membranes in aquatic organisms. In this study, *Daphnia magna* was used to examine the lethal and sublethal toxicity of three POEA formulations consisting of 5:1, 10:1, and 15:1 average oxide:tallowamine. The formulation consisting of 10:1 was the most acutely toxic with a 48-h LC_{50} value of 97.0 ug/L and 15:1 was least toxic at 849.4 lg/L. All formulations inhibited growth at concentrations between 100 and 500 ug/L.

MATERIALS AND METHODS

1. Test material:

Test item: Surfonic® T-5, T-10 and T-15 surfactant

Active substance(s): Polyethoxylated tallowamine (POEA)

Adjuvant/surfactant: The test item, Surfonic®, itself is the adjuvant and generally used with "Roundup-Ready" (a.s. glyphosate) while in this study surfactant was applied purely.

Description: POEA is characterized by the mass ratio between the oxide and tallowamine portions of the molecule. Average oxide : tallowamine ratios were 5:1 (T-5), 10:1 (T-10) and 15:1 (T-15).

Source of test substance: Huntsman International LLC (Salt Lake City, UT, USA)

Lot/Batch #: Not stated

Purity: 98.6%, 99.8% and 99.4% pure for T-5, T-10 and T-15, respectively

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Daphnia magna*

Age of test organisms at study initiation: < 24 hours old

Source: Not stated

Holding conditions prior to test: Waterfleas were cultured in synthetic freshwater according to EPA-821-R-02-012 (USEPA 2002) with pH = 7.43, hardness 84 mg/L and alkalinity 60 mg/L (both as $CaCO_3$). They were fed with yeast and trout chow (Cerophyll®) mixture and maintained under 16:8 light:dark cycle at 25 ± 1 °C until

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[56] Quoted from article

toxicity testing.

4. Test system:

Study type: Static

Guideline: None; study design comparable to OECD 202

GLP: No

Guideline deviations:
- No analytics for actual test item concentration.
- Hardness of medium is below recommendations.
- Temperature was above the range of 18 - 22 °C.
- No first immobility check at 24 h.

Duration of study: 48 h

Test conditions: Tests were conducted in 250 ml beakers filled with 200 ml solution. Measurements of parameters after 48 h while immobilised daphnids were defined dead.

Treatments: 3 different POEA formulation (i.e. T-5, T-10, T-15) treatments and 3 untreated control.

Test concentrations: 7 test concentrations in each treatment: 0.1, 10, 100, 500 1,000 and 10,000 µg/L.

Replicates per concentration: 3

Organisms per replicate: 10

Feeding: None during testing.

Parameters measured: Mortality and growth ratio of surviving individuals at 48 hours exposure. Growth ratio was the average body length in treatments divided by the average body length in.
Data were evaluated using logit analysis and one-way ANOVA with Dunnett or Tukey post-hoc where appropriate (R ver. 2.2.1).

Analytical determination of test concentrations: Not measured

5. Environmental conditions:

Test medium: Synthetic fresh water according to EPA-821-R-02-012 (USEPA 2002).

Temperature: 25 ± 1 °C

Photoperiod: 16 h light

pH: 6.4 – 6.7

Oxygen saturation: 7.93 – 8.41 mg/L ± 0.04

Conductivity: Not stated

Hardness: 84 mg $CaCO_3$/L

Alkalinity: 60 mg $CaCO_3$/L

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:
- A number of deviations from the common OECD guideline lowered the reliability of the study.
- Most critical is the lack of analytical quantification of

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test concentrations, so that endpoints are only based on nominal concentrations.

- Source of daphnid culture is not reported.
- Separation factor of test concentration is higher than recommended.
- A difference of one order of magnitude between the acute toxicity of the 10 EO and 15 EO materials is far greater than would be predicted using established QSAR models for this class of surfactants. This result questions the validity and interpretation of the results from this study. The rank order of EO toxicity is backwards, which impacts the validity of the author's conclusions.

2. Relevance of study: **Not relevant**

Comment: Duration and provision of food is crucial for toxicity testing on *Thamnocephalus platyurus*. An assessment of mortality at 48 h instead of 24 h exposure does not allow conclusions to the stressors' effects.
The manufacturer of the Thamnotoxkit F recommends a maximum exposure duration of 24 hours, as neonates will not survive for more than 24 hours at good health without food supply. This renders the study 'not reliable'.

3. Klimisch code: **Klimisch rating of 3 and not adequate for risk assessment.**

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Extended comments

The German Authority (UBA) has raised a concern about the presumed high toxicity of POEA surfactants to the aquatic invertebrate *Thamnocephalus platyurus* (a freshwater fairy shrimp) based on a publication by Brausch and Smith (2007). Below we provide compelling evidence why this likely is not the case.

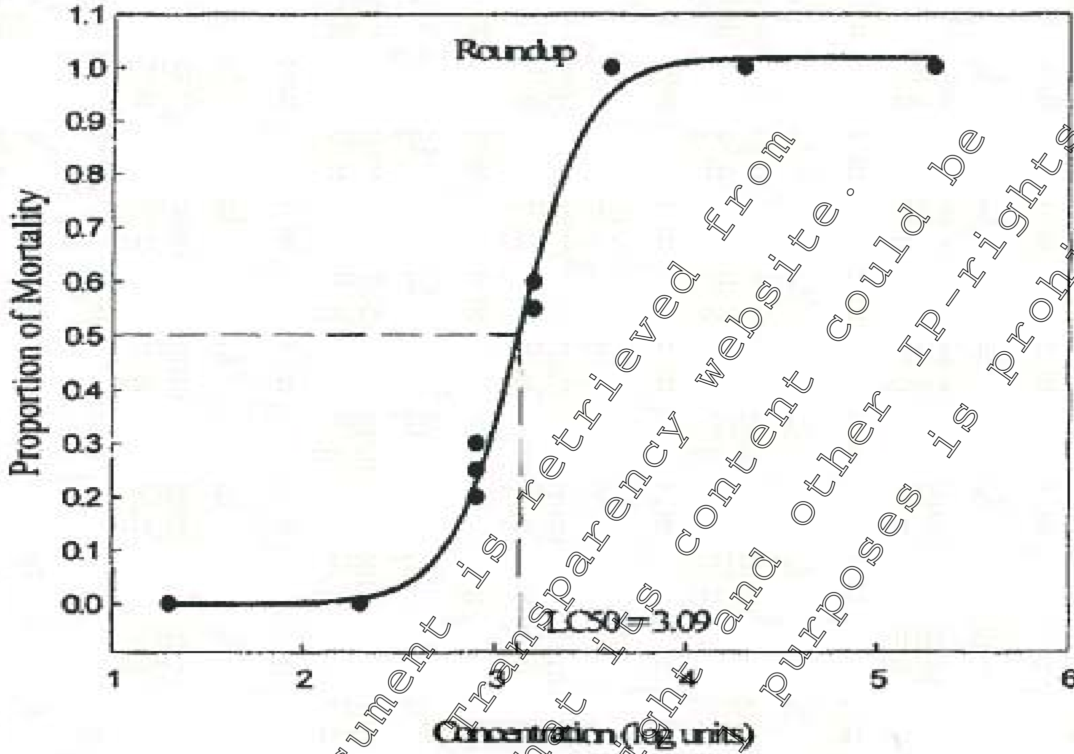
Brausch and Smith (2007) assessed the acute toxicity (48-h LC_{50}) of three POEA surfactants (Surfonic T-5, T-10, and T-15 with 5:1, 10:1, and 15:1 ratios of oxide:tallowamine, respectively) to the aquatic invertebrate *T. platyurus*. The authors conclude that the mean LC_{50} values ranged from approximately 2-5 µg/L. However, neither **the LC50 values**, **the shape of the dose-response curve** nor **the order of magnitude of effect** is consistent with the author's own published data (Brausch et al., 2006) or data from other studies with other aquatic invertebrates (Giesy 2000).

Comparison of LC50 values from Brausch et al. 2006 and Brausch and Smith 2007: The LC50 value for *T. platyurus* exposed to Roundup® Super Concentrate (RU) in Brausch et al. 2006 was reported to be 1.25 mg RU/L, which is similar to LC50 values for other freshwater invertebrates exposed to either RU or POEA surfactant (Giesy 2000). The LC_{50} value for *T. platyurus* exposed to POEA surfactants in Brausch and Smith (2007), however, was reported to be 2-5 µg POEA/L, which is nearly 2 orders of magnitude more toxic than Brausch et al. 2006 when corrected for POEA surfactant concentration. The only logical explanations for this are:

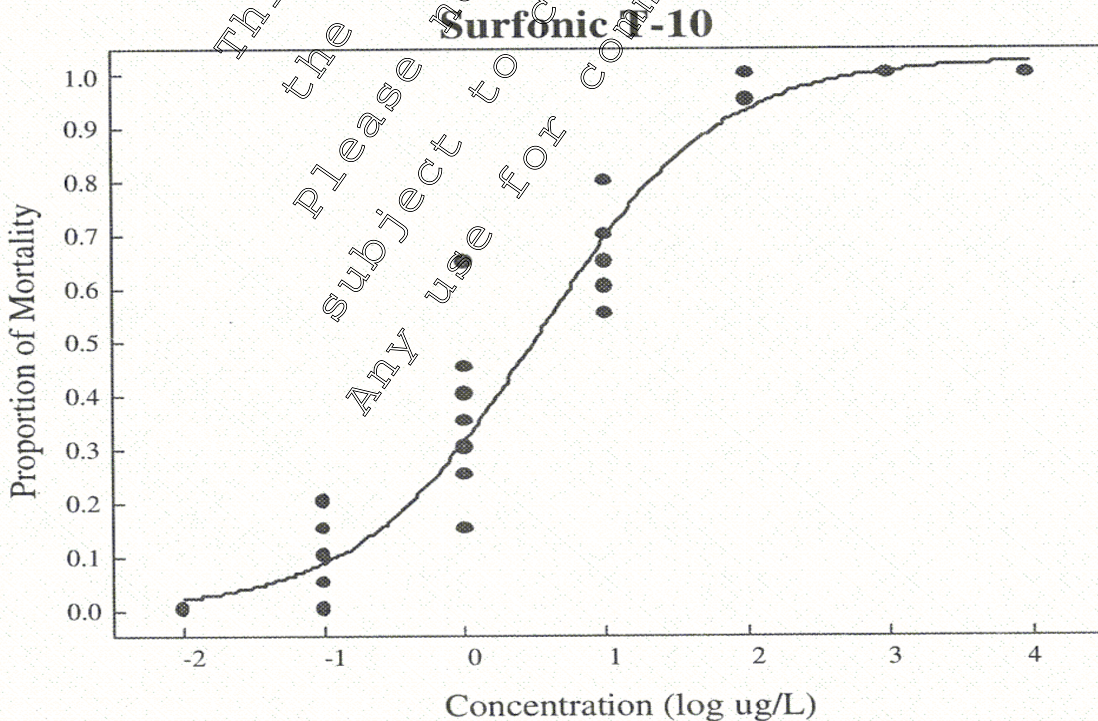
1. There was a mistake made in calculating the LC50 values in the Brausch and Smith (2007) paper.
2. Other components of the Roundup formulation used in Brausch et al. (2006) somehow buffer the effects of the surfactant.[57]
3. The POEA surfactants tested in Brausch and Smith (2007) are not similar to the POEA surfactants used in the Roundup formulation in Brausch et al. (2006).

[57] This possibility is considered unlikely since this has not been observed by other investigators who directly compared POEA and POEA containing glyphosate formulations (Giesy et al. 2000)

The shape of the dose-response curve: It is well established that the toxicity of glyphosate formulations containing POEA surfactants is driven by the surfactant and not the active herbicidal ingredient glyphosate (Giesy et al. 2000). The dose-response is generally steep (i.e., the rate of mortality increases rapidly with increasing concentration) when the toxicity of a RU formulation with POEA surfactant is tested against aquatic organisms. A good example of this is found in a paper published by Bausch et al. 2006 (see below).



Note that all of the mortality of *T. platyurus* exposed to Roundup occurs within one log unit (approximately 2.5 to 3.5). In Brausch and Smith (2007), however, the dose-response is very protracted with mortality of *T. platyurus* exposed to the T-10 POEA surfactant occurring over approximately 3 log units (approximately -1 to 2).



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The order of magnitude of effect: Brausch and Smith (2007) suggest that the LC50 values obtained in their study were in the low µg/L range. However in the introduction of their paper (p. 217), the authors state that, "Three recent studies (Lindgren and Sjostrom 1994; Lindgren et al. 1996; Uppgard et al. 2000) demonstrated that AEOs are very toxic (LC50 values = 1.9-166.0 mg/L) to the freshwater fairy shrimp, *Thamnocephalus platyurus*." Later, in the Results and Discussion section (p. 219), the authors state, "Uppgard et al (2000) used 15 different AEOs for toxicity testing on *T. platyurus* and found 24-h LC50 values ranging from 19-166 µg/L." There are several issues with these two statements:

1. The first statement is reported in mg/L, the second in µg/L. Both are incorrect (see point 5 below).
2. The range of LC50 values in the first statement is 1.9-166.0 **mg/L** and the other statement is 19-166 **µg/L**. Even if we attribute the ranges to an error in decimal placement, the units do not match and the significant figure issue is of interest. Neither Lindgren et al. 1996 nor Uppgard et al. 2000 report a value of 166 or 166.0. However, Krogh et al. 2003 (summarizing Lindgren et al. 1996 and Uppgard et al. 2000) reports a range of 1.9-166.0. Uppgard et al. 2000 reports his triplicate values that had been reported earlier in Lindgren et al. 1996 of 194.00, 144.00, and 158.00 for a mean value reported in Lindgren et al. 1996 (but not Uppgard et al. 2000) of 165.3. Therefore, Brausch and Smith (2007) appear to have used the data as reviewed in Krogh et al. 2003, but attributed the slightly modified value to Lindgren et al. 1996 and Uppgard et al. 2000.
3. While the Lindgren and Sjöström (1994) paper was about non-ionic surfactants, the paper provided no information on toxicity studies with *T. platyurus* as indicated in Brausch and Smith (2007).
4. Table 1 of Brausch and Smith appears to have a number of inconsistencies. The tallow amine surfactants Surfonic T-5, T-10, and T-15 are indicated to have only 3-5 alkyl carbons per molecule. This would be very unusual since the alkyl chain lengths of the fatty amines derived from animal fat typically have primarily 16-18 carbon chain lengths. The number of oxide carbons relative to alkyl carbons and tallow to oxide ratio by mass also seem to be in error.
5. Neither Lindgren et al. 1996 nor Uppgard et al. 2000 presented their toxicity data (LC50 values) in µg/L or mg/L. Instead they presented their data in µM. Brausch and Smith (2007) appear to have mistakenly changed the units from µM to mg/L or µg/L without the appropriate conversion using the molecular weight. Given a molecular weight range of approximately 500-1000, the error represents a 2-3 order of magnitude misrepresentation of the Lindgren et al. 1996 and Uppgard et al. 2000 data.

The points above raise doubt as to the actual units of the LC50 values reported from the tests of Brausch and Smith (2007). Did the authors understand the distinction between micrograms per liter (µg/L) and micromoles per liter (µM)? If the LC50 values reported by Brausch and Smith were actually in units of micromoles per liter, the corresponding values in mass per liter would be 1.8 to 2.5 mg/L. Were the actual surfactants tested really comparable in alkyl chain length to tallowamine surfactants utilized in glyphosate formulations?

Taken as a whole, this summary provides strong evidence that the Brausch and Smith (2007) paper overestimates the toxicity of POEA surfactants used in RU formulations to freshwater invertebrates and should not be used for risk assessment purposes.

Authors	Year	Study title
Bringolf, R.B., Cope, W.G., Mosher, S., Barnhart, M.C., Shea, D.	2007	Acute and chronic toxicity of glyphosate compounds to glochidia and juveniles of *Lampsilis siliquoidea* (Unionidae) Environmental Toxicology and Chemistry Volume: 26 Number: 10 Pages: 2094-2100 URL: http://onlinelibrary.wiley.com/doi/10.1897/06-519R1.1/abstract DOI: 10.1897/06-519R1.1 ISSN: 0730-7268 (Print)

Abstract[58]

Native freshwater mussels (family Unionidae) are among the most imperiled faunal groups in the world. Factors contributing to the decline of mussel populations likely include pesticides and other aquatic contaminants; however, there is a paucity of data regarding the toxicity of even the most globally distributed pesticides, including glyphosate, to mussels. Therefore, the toxicity of several forms of glyphosate, its formulations, and a surfactant (MON 0818) used in several glyphosate formulations was determined for early life stages of *Lampsilis siliquoidea*, a native freshwater mussel. Acute and chronic toxicity tests were performed with a newly established American Society of Testing and Materials (ASTM) standard guide for conducting toxicity tests with freshwater mussels. Roundup_, its active ingredient, the technical-grade isopropylamine (IPA) salt of glyphosate, IPA alone, and MON 0818 (the surfactant in Roundup formulations) were each acutely toxic to *L. siliquoidea* glochidia. MON 0818 was most toxic of the compounds tested and the 48-h median effective concentration (0.5 mg/L) for *L. siliquoidea* glochidia is the lowest reported for any aquatic organism tested to date. Juvenile *L. siliquoidea* were also acutely sensitive to MON 0818, Roundup, glyphosate IPA salt, and IPA alone. Technical-grade glyphosate and Aqua Star were not acutely toxic to glochidia or juveniles. Ranking of relative chronic toxicity of the glyphosate-related compounds to juvenile mussels was similar to the ranking of relative acute toxicity to juveniles. Growth data from chronic tests was largely inconclusive. In summary, these results indicate that *L. siliquoidea*, a representative of the nearly 300 freshwater mussel taxa in North America, is among the most sensitive aquatic organisms tested to date with glyphosate-based chemicals and the surfactant MON 0818.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Roundup Ultramax ™, Glyphosate IPA salt containing POEA Aqua Star®, Glyphosate IPA salt without POEA Glyphosate acid (technical) Glyphosate IPA salt (technical) Isopropylamine (IPA) (acute toxicity testing only) MON 0818
Active substance(s):	Glyphosate acid, IPA, POEA
Surfactant:	MON 0818 (Polyethoxylated tallow amine (POEA))
Description:	none
Source of test substance:	Roundup Ultramax ™: Retailer (unspecified) Aqua Star®: Retailer (unspecified) Glyphosate acid (technical): Chem Service, West Chester, PA

[58] Quoted from article

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Glyphosate IPA salt (technical): Chem Service, West Chester, PA
Isopropylamine (IPA): Sigma Aldrich, St. Louis, MO
MON 0818 (Polyethoxylated tallow amine (POEA)): Monsanto Company, St. Louis, MO

Lot/Batch #: Not stated

Purity: Roundup Ultramax ™: 50.2% glyphosate IPA salt
Aqua Star®: 53.8% glyphosate IPA salt
Glyphosate acid (technical): 98%
Glyphosate IPA salt (technical): >95%
Isopropylamine (IPA): 99.5%
MON 0818: not stated (proprietary commercial blend of POEA surfactants)

Stock solution: A working solution of IPA (10 000 mg/L) was prepared by dissolving 100 mg of neat IPA in deionized water to a final volume of 10 ml. The working solution of MON 0818 (10,000 mg/L) was prepared by dissolving 250 mg of MON 0818 in deionized water to a final volume of 25 ml.

2. Vehicle and/or positive control: Young of year largemouth bass (*Micropterus salmoides*) for transformation of glochidia to juveniles.

3. Test organism:

Species: *Lampsilis siliquoidea*

Age of test organisms at study initiation: Glochidia
Juveniles:
Acute tests: one week post transformation for glyphosate IPA and Roundup; one month post transformation for technical-grade glyphosate, IPA, and Aqua Star; and two months post transformation for the MON 0818 test.
Chronic tests: one month post transformation for technical-grade glyphosate and Roundup and two months post-transformation in tests for Aqua Star, glyphosate IPA, and MON 0818.

Data are not completely consistent to the data provided in the test organism description:
Juveniles (acute tests) 2 (average shell length 732 ± 96 µm) to 8 weeks post-metamorphosis (average shell length 2196 ± 432 µm).

Juveniles (chronic tests) 3(mean shell length 1012 ± 118 µm) to 8 weeks post-metamorphosis (average shell length 2057 ± 416 µm).

Source: Glochidia: Brooding adult female *L. siliquoidea)* were obtained from Silver Fork of Perche Creek, Boone County, MO and transferred in chilled coolers for harvest of glochidia to Missouri State University, MO. Glochidia were harvested from marsupial gills of at least 3 females. Glochidia were then shipped in chilled coolers to North Carolina State University, Raleigh, NC.
Juveniles: largemouth bass were infested with glochidia by swimming for 15 min in a suspension containing approx. 4000 viable glochidia/L. Following transformation, juveniles were recovered from host fish and transferred to a culture system (Barnhart, 2006).
Largemouth bass: Missouri Dept. of Conservation Chesapeake Hatchery, Mount Vernon, MO

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Holding conditions prior to test: Viability was assessed exposing three subsamples of approximately 50 - 100 glochidia each to saturated NaCl solution. Specimens were used for toxicity tests only if initial viability was ≥90%.
Juveniles were held at 22 to 23 °C with weekly water replacements.

Acclimatisation: Glochidia were acclimatised for 2 h in a 50:50 mixture of culture: dilution water prior to testing.
Juveniles were acclimatised for 24 h by tempering into dilution water.

4. Test system:

Study type: 48 h static, acute for glochidia
96 h static renewal for juveniles (acute),
21- or 28 d static renewal (48 or 72 h intervals (chronic))

Guideline: ASTM E2455-06 (2006)

GLP: No

Guideline deviations: pH was slightly greater than range required for reconstituted hard water (7.8 – 8.0)

Duration of study: Glochidia: 48 h (acute)
Juveniles: 96 h (acute)
Juveniles 21 or 28 d (chronic)

Test conditions: Glochidia were exposed in 150 mL glass beakers filled with 100 mL of reconstituted hard water (ASTM 2006). After 24 and 48 h, shell closure response was evaluated at ×10 to 20 magnification (NaCl response test).
Juveniles for acute testing were kept in 90 × 50 mm glass dishes containing 100 mL test solution. Test solution was renewed (90%) after 48 h. Foot movement within a 5 min period was evaluated at ×20 to 40 magnification (stereomicroscope).

Replicates per concentration: Glochidia acute: 3
Juveniles acute: not stated
Juveniles chronic: not stated

Organisms per replicate: Glochidia acute test: approx. 200 glochidia
Juvenile acute test: 7 to 10 juveniles
Juvenile chronic test: 3 juveniles

Feeding: Stock juveniles were fed with algal suspensions (*Neochloris oleoabundans* and commercial larviculture preparations of *Nannochloropsis*, *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira weissflogii)* (Reed Mariculture, Campbell, CA, USA).
acute tests: no feeding
Chronic tests: Instant algae ®Shellfish Diet and *Nannochloropsis* concentrate

Parameters measured: Glochidia acute test: immobility at 24 and 48 hours (shell closure),
Juveniles acute test: immobility at 48 and 96 h (foot movement)
Juveniles chronic test: mortality at day 7, 14, 21 and 28, except for glyphosate acid (technical), which was terminated after 21 days; mean growth based on shell length in treatments with ≥50% survival.

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Acute test: Temperature, pH, dissolved oxygen, conductivity, alkalinity and hardness at the beginning and end of test in one replicate of at least two treatments.

EC/LC_{50} and 95% confidence intervals were calculated using trimmed Spearman-Karber; growth was evaluated using ANOVA followed by Dunnett's test as post-hoc.

Test concentrations: Acute test: Five or six treatments and control; test concentrations are not further specified.

Chronic test:

Glyphosate acid (technical): 12.5, 50 and 200 mg a.e./L
Glyphosate IPA (technical): 0.8, 1.6, 3.1, 6.3 and 12.5 mg a.e./L
Aqua Star ®: 12.5, 25, 50, 100, 200 mg/L
Roundup ®: 0.6, 1.3, 2.5, 5 and 10 mg/L
MON 0818: 0.3, 0.6, 1.3, 2.5 and 5 mg/L

Analytical determination of test concentrations: Acute test: verified as glyphosate acid (a.e.) by HPLC in each of three replicates of control, low, intermediate and high test concentration at start of exposure. Measured concentrations ranged from 82.2 to 104.4% (mean 94.2%).

Chronic test:

Validity criteria: Glochidia: initial viability ≥90%
Control viability: >90%

5. Environmental conditions:

Test medium: Reconstituted hard water (ASTM E2455-06, 2006)

Temperature: 21.1 – 21.8 °C

Photoperiod: Not stated

Light intensity: Not stated

pH: 8.22 – 8.76

Oxygen saturation: >83%

Conductivity: 500 – 600 µS/cm

Hardness: 158 - 170 mg $CaCO_3$/L

Alkalinity: 114 - 128 mg $CaCO_3$/L

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions** (omissions and inconsistencies in reporting experimental details).

Comment
- Huge differences in age and especially size between tests with different test items may hamper comparability of results (different filtering rate, individual metabolism etc. may result in differences of sensitivity).
- No test concentrations provided for acute tests
- No number of replicates stated for chronic exposure
- Only initial concentrations of acute exposure verified by analytical measurements

2. Relevance of study: **Relevant with restrictions**

3. Klimisch code: Acceptable publication which meets basic scientific principles,

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methodology and basic data given are comparable to ASTM E2455-06 (2006). However, gastropod risk assessment no EU requirement)

This results in a **Klimisch rating of 2**

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	Year	Study title
Chen, C.Y., Hathaway, K.M., Folt, C.L.	2004	Multiple stress effects of Vision® herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands Environmental Toxicology and Chemistry Volume: 23 Isssue: 4 Pages: 823-831

Abstract[59]

As part of a multiple-tier research program, interactions of the herbicide Vision® (glyphosate) with two stressors, pH and food level, were examined. Effects of the formulated product Vision were tested at two test concentrations (0.75 and 1.50 mg acid equivalent/L), two pH levels (pH 5.5 and 7.5), and under high and low food concentrations. Effects of each stressor alone and in combination were examined using two common wetland taxa: Zooplankton, *Simocephalus vetulus*, and tadpoles (Gosner stage 25) of *Rana pipiens*. For *S. vetulus*, survival, reproduction, and development time were measured; survival was measured for *R. pipiens*. For both species, significant effects of the herbicide were measured at concentrations lower than the calculated worst-case value for the expected environmental concentration ([EEC], 1.40 mg acid equivalent/L). Moreover, high pH (7.5) increased the toxic effects of the herbicide on all response variables for both species even though it improved reproductive rate of *S. vetulus* over pH 5.5 in the absence of herbicide. Stress due to low food alone also interacted with pH 5.5 to diminish *S. vetulus* survival. These results support the general postulate that multiple stress interactions may exacerbate chemical effects on aquatic biota in natural systems.

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MATERIALS AND METHODS

1. Test material:

Test item(s):	Vision® (containing 15% MON 0818)
Active substance(s):	Glyphosate isopropylamine salt
Surfactant:	Polyethoxylated tallowamine (POEA)
Description:	For treatment of post-harvest areas in forestry to suppress the growth of undesirable competing vegetation; most commonly used in high-yield spruce/fir plantations.
Source of test substance:	Monsanto Company, Winnipeg, MB, Canada.
Lot/Batch #:	Not stated
Purity:	356 g acid equivalent/L (glyphosate isopropylamine salt)
Stock solution:	Not stated
2. Vehicle and/or positive control:	none
3. Test organism:	
Species:	*Simocephalus vetulus* (Cladocera, Daphniidae) *Rana cascadae* (Anura, Ranidae)
Age of test organisms at study initiation:	*S. vetulus*: 10-days old adult and <24-h old juvenile neonates (2 different experiments). *R. cascadae*: larvae at Gosner stage 25 (G 25)

[59] Quoted from article

Source: *S. vetulus* culture established with wild-caught individuals from forest pond located in Bomoseen State Game Park (western Vermont, USA: 43.66619°N, 073.22845°W).
R. cascadae: eggs and tadpoles from Carolina Biological (Burlington, NC, USA) and from field.

Holding conditions prior to test: *S. vetulus*: kept in filtered lake water (Storrs Pond, New Hampshire, USA; pH = 6.8) added with 5.9 µM disodium ethylenediamene tetraacetate acid (EDTA). Fed with 300 µg/L algae of species *Cryptomonas erosa* daily.
R. cascadae: kept in filtered Storrs Pond water at 20°C on a 14:10-h light:dark regime. Fed with algae *C. erosa*, *Chlamydomonas reinhardi* and Tetramin fish food.

Acclimatisation: Frog tadpoles were acclimated to lab temperature of 20°C for 3 days after collecting from the field, and 24 h before experiment adjusted to pH 5.5 and 7.5, respectively.
Cladoceran experimental females and neonates were produced from the 2nd or 3rd clutch of brood females of approximately the same age.

4. Test system:

Study type: Static renewal (2 day intervals)

Guideline: Not stated

GLP: No

Guideline deviations: Not applicable

Duration of study: *S. vetulus* adult experiments: 8 days
S. vetulus juvenile experiments: terminated when all either died or produced their first clutch.
R. cascadae survival experiments: 10 days

Test conditions: *S. vetulus* adult experiments: in capped 145-ml jars containing filtered pond water rotated on plankton wheel on a cycle of 2 min rotation and 15 min stationary (1 revolution per 100 s). Survival was measured daily.
S. vetulus juvenile experiments: in 40-ml glass vials without rotation. Survival was measured daily.
R. cascadae survival experiments: in 250-ml under environmental conditions described below. Survival was measured daily.

Treatments: 2 pH levels (5.5 and 7.5), 2 food levels (cladocerans: 300 and 800 µg C/L; tadpoles: 40 and 800 µg C/L) 2 test concentrations, and untreated control.

Test concentrations: 0.75 and 1.5 mg formulation/L

Replicates per treatment: 12 (*S. vetulus* adult)
10 (*S. vetulus* juvenile)
8 (*R. cascadae* survival)

Organisms per replicate: 1 (*S. vetulus* adult)
1 (*S. vetulus* juvenile)
1 (*R. cascadae* survival)

Feeding during experiments: Algae *Chlamydomonas reinhardi*

Parameters measured: *S. vetulus* adult experiments: survival and reproduction.
S. vetulus juvenile experiments: development and survival.
R. cascadae survival experiments:survival.

Survival and development parameters were analyzed by log-

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	rank test and reproduction parameters by ANOVA.
Analytical determination of test concentrations:	Verified by gas chromatography and were found to be within 20% of nominal concentrations.
Validity criteria:	Not applicable

5. Environmental conditions:

Test medium:	Filtered pond water from Storrs Pond (New Hampshire, USA) with adjusted pH by Beckman electrodes.
Temperature:	20 °C
Photoperiod:	14 h
Light intensity	43.8 µmol/s m^3/ua
pH:	5.5 7.5
Oxygen saturation	93%
Conductivity:	140 L/Ω
Hardness:	Not stated
Alkalinity:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment

- Acceptable, well-documented publication which meets basic scientific principles.
- Sound statistical and experimental design.
- Natural pond water was used for experiments complicating interpretation of results and allows only general conclusions with restrictions.

2. Relevance of study: **Relevant**

Comment:

Study performed not with the lead formulation under evaluation.

Test medium is filtered lake water from Storrs Pond (New Hampshire, USA) which is a swimming lake of Darthmouth College. Even though water was filtered and medium was prepared prior to experiments it can not be excluded that water still contains additional stressors. This may lead to an increased variability of glyphosate and surfactant effects on organisms.

However, this study still matches all scientific principles in terms of experimental design and statistical analysis. The study produced, therefore, relevant data of effects on aquatic organisms while it is mentioned in the article that these effects are rather to be addressed to the surfactant POEA than to the isopropylamine salt of glyphosate.

3. Klimisch code: **Klimisch rating of 2**

Author(s)	Year	Study title
Kaiser Dutra, B., Amorim Fernandes, F., Motta Failace, D., Turcato Oliveira, G.	2006	Effect of roundup® (glyphosate formulation) in the energy metabolism and reproductive traits of *Hyalella castroi* (Crustacea, Amphipoda, Dogielinotidae). Ecotoxicology Volume: 20 Pages: 255-263 DOI: 10.1007/s10646-010-0577-x ISSN: 0963-9292 (Print), 1573-3017 (Online)

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Abstract[60]

Roundup® (glyphosate formulation) is a nonselective and posts emergent herbicide used for controlling aquatic weeds and different concentrations are used in cultures around the world. The objective of this investigation was to examine the effects of Roundup® (glyphosate formulation) on the biochemical composition, levels of lipoperoxidation, Na^+/K^+ATPase activity and reproductive traits in the *Hyalella castroi*. Amphipods were collected in summer 2009, in the southern Brazilian highlands. In the laboratory, the animals were kept in aquariums under controlled conditions for 7 days, and after this period they were exposed to 0.36, 0.52, 1.08 and 2.16 mg/l of glyphosate for 7 days. After the period of exposure, the animals were immediately frozen for determination of glycogen, proteins, lipids, triglycerides, cholesterol, levels of lipoperoxidation, and Na^+/K^+ATPase activity. During each day of the cultivation reproductive traits (number of reproductive pairs, ovigerous females and eggs in the marsupium) were observed. All concentrations of Roundup® induced significant decreases in all biochemical parameters and Na^+/K^+ATPase activity, and significant increase in lipoperoxidation levels. Showing this form a potentially toxic effect at very low concentrations, this pattern of results can lead to significant changes in trophic structure of limnic environments because these amphipods are important links in food chain in these habitats.

MATERIALS AND METHODS

1. Test material:

Test item:	Unknown formulation of Roundup ® and not MON 52276
Active substance(s):	Glyphosate IPA salt
Surfactant:	Stated to be POEA but unable to confirm
Description:	Not stated, not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested. No a.s. loading reported.
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	Not stated

2. Vehicle and/or positive control: None

3. Test organism:

Species:	*Hyalella castroi*
Age of test organisms at study initiation:	adult
Source:	Collected in Sao José dos Ausentes Municipality, Rio Grande

[60] Quoted from article

do Sul, Brazil together with *Callitriche rimosa*.

Holding conditions prior to test: Specimens were kept for 7 days separated by gender in aerated 20 L aquaria and fed daily *at libitum* with a combination of commercial fish feed and *C. rimosa*.

4. Test system:

Study type: Static

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 7 d

Test conditions: Tests were conducted in 20 L aquaria, origin of water not stated.

Replicates per concentration: Experiment was repeated 3 times on different months

Organisms per replicate: 10 couples (20 specimens)

Feeding: fed daily *at libitum* with a combination of commercial fish feed and *C. rimosa*.

Parameters measured: Daily for mortality, number of reproductive pairs, ovigerous females and number of eggs in the brood pouch. Glycogen, protein, lipid, triglyceride, cholesterol, lipoperoxidation levels and Na+/K+ATPase activity were determined in quadruplicate on total homogenates at the end of exposure.
Data were evaluated using one-way ANOVA followed by a Bonferroni test, for comparison between sexes, a two-way ANOVA was used.

Test concentrations: 0.36, 0.52, 1.08 and 2.16 mg glyphosate/L (nominal) and an untreated control

Analytical determination of test concentrations: Not stated

5. Environmental conditions:

Test medium: Not reported

Temperature: 23 ± 1 °C

Photoperiod: 12 h light

pH: Not stated

Oxygen saturation Not stated

Conductivity: Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Test medium description missing, pH and oxygen content not stated; i.e. an indirect effect caused by decrease of pH only cannot be excluded.
- Renewal/cleaning of tanks or change of water not reported
- No ‘real’ replicates, experiment was repeated three times at different months, but differences between

repetitions are not reported.

- Description of exposure is confusing; within the experimental procedure paragraph, it is stated that animals were acclimated for 7 days, later on it is stated that animals were kept in the laboratory for 24 hours prior to start of exposure/experiments.
- Clear description of the test substance missing; not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
- Confirmation of glyphosate concentrations in water was not performed. This omission greatly reduces the reliability of this study for use in an aquatic risk assessment.

2. Relevance of study: **Not relevant**

Comment: It is not evident what formulation was tested and no analytical confirmation of the test solution was performed. Critical environmental parameters to help judge the validity of the study were not reported. Exposure concentrations greatly exceed environmentally realistic concentrations.

3. Klimisch code: This renders the study 'not reliable', resulting in a **Klimisch rating of 3 and not acceptable for risk assessment.**

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Author(s)	Year	Study title
Le, T.-H., Lim, E.-S., Lee, S.K., Choi, Y.-W., Kim, Y.-H., Min, J.	2010	Effects of glyphosate and methidathion on the expression of the Dhb, Vtg, Arnt, CYP4 and CYP314 in *Daphnia magna* Chemosphere Volume:79 Issue: 1 Pages: 67-71 DOI: 10.1016/j.chemosphere.2009.12.067 ISSN: 0045-6535

Abstract[61]

In this study, the expression of five stress responsive genes was quantified and analyzed using a semi-quantitative RT-PCR to study the changes in their expression in *Daphnia magna* after exposure to known pesticides, glyphosate and methidathion. Hemoglobin (*Dhb*), which was used to show the effect of the oxygen level in the aquatic system, was significantly expressed in *D. magna* after exposure to glyphosate and methidathion. Additionally, aryl hydrocarbon receptor nuclear translocator (*Arnt*), a gene related to the metabolism of aryl hydrocarbons, had lower expression levels in *D. magna* than within the control. *CYP4*, which was used among cytochrome P450s (CYPs) to show the effects on the fatty acid and steroids metabolisms, was down-regulated in *D. magna* exposed to glyphosate. However, methidathion affected the expression of *CYP314*, which was used to show effects of ecdysis, not *CYP4* in *D. magna*. Therefore, glyphosate and methidathion probably caused physiological effects with different patterns in *D. magna*, especially metabolisms related to CYPs. On the other hand, only vitellogenin (*Vtg*), which was responsive to the estrogenic potency, did not show any differences in *D. magna* after exposure to glyphosate or methidathion.

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate obtained through Fluka, US but no additional information

Active substance(s): Not stated

Adjuvant / Surfactant: Not stated

Description: none

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: Not stated

Stock solution: Not stated

2. Vehicle and/or positive control: None; but test was also conducted exposing *D. magna* to methidathion (organophosphate insecticide, banned in the EU)

3. Test organism:

Species: *Daphnia magna*

Age of test organisms at study initiation: Neonates < 24 h generated by mother organisms less than 30 d old.

Source: Korea Institute of Toxicology (Daejeon, Korea)

Holding conditions: According to US EPA 2002; at 20 ± 1 °C in 2 L beakers

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[61] Quoted from article

	containing 1.5 L of hard reconstituted water (HRW), containing 0.12 g $MgSO_4$/L, 0.192 g $NaHCO_3$/L, 0.008 g KCl/L, 0.12 g $CaCO_3$/L in deionized water. HRW was aerated for 24 h and controlled at a pH of 8.2 ± 0.2, photoperiod of 16 h and loading of 30-50 specimens /2 L. Three times a week medium was renewed and specimens were fed with mix of *Pseudokirchneriella subcapitata* and YTC (Aquatic Biosystem Inc., Colorado, U.S.).
Acclimatisation:	24 h with no feeding under test conditions

4. Test system:

Study type:	Static toxicity
Guideline:	US EPA-821-R-02-012 (2002)
GLP:	Not stated
Guideline deviations:	-
Duration of study:	24 h
Test conditions:	Acute toxicity tests were conducted in 50 ml medium, exposure to analyse gene expression was conducted in 500 ml chambers filled with 200 ml test solution.
Replicates per treatment:	3
Organisms per replicate:	10
Feeding:	none
Parameters measured:	Acute toxicity test: mortality after 24 h. Relative expression levels of five genes (Dhb, Vtg, Arnt, CYP4 and CYP314) by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).
Test concentrations:	<u>Acute test:</u> concentrations not specified; acute test was used to determine LC_5, LC_{10}, LC_{20} and LC_{50} values for subsequent exposure to analyse gene expression. <u>Gene expression exposure:</u> 190, 202, 214, and 234 mg glyphosate/L
Analytical determination of test concentrations:	none

5. Environmental conditions:

Test medium:	Medium not explicitly specified; it may be assumed that the definite tests were conducted using the same (HRW) medium as used for culturing daphnids.
Temperature:	20 ± 1 °C
Photoperiod:	16 h light
Light intensity:	Not stated
pH:	Not stated
Dissolved oxygen:	Not stated
Conductivity:	Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

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Comment:

- Many relevant experimental details not stated. Especially it is of utmost importance that neonates of daphnids are not of first brood of stock specimens (see e.g. OECD 202). With regard to test medium, one may speculate that for the exposures the same medium was used as for the culturing, but a distinct confirmation is lacking. pH values and oxygen concentration are not stated.
- Unclear, whether endpoints reported refer to mg active substance/L or mg acid equivalent/L.

2. Relevance of study: **Not relevant**

Comment: It is unclear from the paper what form of glyphosate was tested exactly. The LC_{50} value reported following the 24 h acute exposure is substantially higher than the endpoint used for the ecological risk assessment for glyphosate acid and was reported to be 234 mg/L. Even the lowest concentration tested is far above concentration considered for actual ecological risk assessment and far above environmentally relevant concentrations. Only semi-quantitative PCR was used in these assays and the validity of the methodology to evlaute mRNA levels is absent. The ascertain that glyphosate would impact ARNT mRNA levels is based on a false premise, glyphosate is not an aryl hydrocarbon, and that data shows far less than a 2-fold change in expression. Changes less than 2-fold with this coarse semi quantitative methodology must change by at least 2-fold for qualitative conclusion of an effect on gene expression. Any impact on CYP4 and ARNT expression represents frank toxicity. It must be recognized that significant impacts on CYP4 expression were only reported at very high LCx levels and waterborne concentrations. Consequently, the decrease in transcript levels reflects frank toxicity. This renders the study not relevant for an ecological risk assessment.

3. Klimisch code: **Klimisch rating of 3 and not relevant for risk assessment.**

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Author(s)	Year	Study title
Mensah, P.K., Muller, W.J., Palmer, C.G.	2011	Acute toxicity of Roundup® herbicide to three life stages of the freshwater shrimp *Caridina nilotica* (Decapoda: Atyidae) Physics and Chemistry of the Earth Volume: 36 Issue: 14-15 Pages: 905-909 Url: http://www.sciencedirect.com.scopeesprx.elsevier.com/science/article/pii/S1474706511001872 DOI: 10.1016/j.pce.2011.07.071 ISSN: 14747065

Abstract[62]

Glyphosate based herbicides, including Roundup®, are frequently used in the chemical control of weeds and invading alien plant species in South Africa. These herbicides ultimately get into water courses directly or indirectly through processes such as drifting, leaching, surface runoff and foliar spray of aquatic nuisance plants. Despite their widespread use, no water quality guideline exists to protect indigenous South African freshwater organisms from the toxic effects of these herbicides. The toxicity of the herbicide Roundup® was assessed using three different life stages of the freshwater shrimp *Caridina nilotica*, a prevalent species in South African freshwater ecosystems. Neonate (<7days post hatching (dph)), juvenile (>7dph and <20dph) and adult (>40dph) shrimps were exposed to varying concentrations (1.5-50mg/L acid equivalence (a.e.)) of the herbicide in 48 and 96h acute toxicity tests in order to determine the most sensitive life-stage. The results showed neonates to be more sensitive to Roundup® than both juveniles and adults with mean 96h LC_{50} values of 2.5, 7.0 and 25.3mg/L a.e. respectively. The estimated 96h LC_{50} of neonates is much lower than the application rate (20-30mg/L a.e.), although the application's impact will depend on the dilution rate of the applied concentration in the environment. All three life-stages of unexposed animals exhibited active and coordinated movement but exposed shrimps were erratic and slow in their movements, with neonates showing most of these behavioral irregularities. This study shows that low levels of the herbicide Roundup® may adversely affect *C. nilotica* health and survival. Thus, the herbicide should be carefully managed to minimize any negative impact on non-target freshwater organisms.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup ®
Active substance(s):	Glyphosate IPA salt
Surfactant:	Polyethoxylated tallowamine (POEA)
Description:	Not stated, not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
Source of test substance:	Monsanto South Africa (Pty) Ltd.
Lot/Batch #:	Not stated
Purity:	360 g a.e./L
Stock solution:	2% stock solution was prepared by dissolving 20 mL Roundup® with distilled water to the 1000 mL mark, resulting in a stock concentration of 700 mg a.e./L.

[62] Quoted from article

2. Vehicle and/or positive control: None

3. Test organism:

Species:	*Caridina nilotica*
Age of test organisms at study initiation:	3 different developmental stages exposed: neonate (<7 days post hatching), juvenile (>7 and <20 days post hatching) and adults (> 40 days post hatching)
Source:	Culture maintained at Unilever Center for Environmental Water Quality, Grahamstown. South Africa
Acclimatisation:	24 h in a climate-controlled environment (24 ± 1 °C and 14 h light)

4. Test system:

Study type:	Static
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	48 and 96 h
Test conditions:	Not specified
Replicates per concentration:	3
Organisms per replicate:	10
Feeding:	None
Parameters measured:	Dead shrimps were recorded twice daily. Swimming behaviour was observed concurrently. Temperature, conductivity, pH and dissolved oxygen were recorded daily. Mortality data were evaluated using probit analysis. One-way ANOVA followed by Newman-Keuls multiple comparison test was used to compare mean LC_{50} values of all age groups Student's t-test to compare mean LC_{50} values of separate age groups.
Test concentrations:	0, 1.7, 2.6, 4.1, 6.4 and 8 mg a.e./L (neonates) (1st run) 0, 1.3, 2.1, 3.3, 5.1 and 8 mg/L (neonates) (2nd run) 0, 1.7, 2.6, 4.1, 6.4, 8 and 10 mg a.e./L (juveniles) (1st run) 0, 1.3, 2.1, 3.3, 5.1, 8 and 10 mg/L (juveniles) (2nd run) 0, 5.4, 8.4, 13.1, 20.5, 32 and 50 mg a.e./L (adults) (1st run) 0, 4.3, 6.7, 10.5, 16.4, 25.6 and 40 mg/L (adults) (2nd run)
Analytical determination of test concentrations:	Not measured

5. Environmental conditions:

Test medium:	Dechlorinated tap water
Temperature:	24 ± 1 °C
Photoperiod:	14 h light
Light intensity	Not stated
pH:	8.5 ± 0.5
Oxygen saturation	5.9 ± 0.3
Conductivity:	0.9 ± 0.05 mS/cm
Hardness:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions** (omissions in material and methods).

Comment:
- Clear description of the test substance missing; not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
- Test conditions (loading etc.) not specified in detail

2. Relevance of study: **Relevant with restrictions**

Comment: A clear description or specification of the test substance is missing, especially with regard to adjuvants and surfactants.

3. Klimisch code: This renders the study 'reliable with restrictions', resulting in a **Klimisch rating of 2.**

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Author(s)	Year	Study title
Sarigül, Z., Bekcan, S.	2009	Acute toxicity of the herbicide glyphosate on *Daphnia magna* Tarim Bilimleri Dergisi Volume: 15 Issue: 2 Pages: 204-208 Publication not available online DOI: not applicable ISSN: not found

Abstract[63]

In this study, median lethal concentrations (LC_{50}) of herbicide Roundup, which contains %48 glyphosate, on *Daphnia magna* for 24 an 48 hours were determined. The experiment has been conducted with the method of static bioassay on two series, five different concentrations (0.0115; 0.018; 0.021; 0.028; 0.032) and one control group have been used. The period of the experiment is 24 and 48 hours. As to the LC_{50} values have been calculated with the method of probit analysis. Experimental results showed that; the concentration of the glyphosate which killed 50 % of *Daphnia magna was* 0.019 mg/L (%95 confidence interval=0.012 mg/L–0.024 mg/L) for 24 hours, but the concentration of the glyphosate which killed 50 % of *Daphnia magna was* 0.012 mg/L (%95 confidence interval=0.001 mg/L-0.016 mg/L) for 48 hours.

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MATERIALS AND METHODS

1. Test material:

Test item: Roundup® (not specified further)
Active substance(s): Not stated
Surfactant: Not stated
Description: Colourless crystals
Source of test substance: Not stated
Lot/Batch #: Not stated
Purity: 48% glyphosate

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Daphnia magna*
Age of test organisms at study initiation: Not stated
Source: Kepez Aquaculture Research Institute
Acclimatisation: Not stated

4. Test system:

Study type: Static
Guideline: None; study design comparable to OECD 202
GLP: No
Guideline deviations:
- Oxygen concentration was below validity criteria
- Test medium is not specified

[63] Quoted from article

- According to OECD 202 it is strongly recommended that specimens are not first brood progeny.

Duration of study: 48 h

Test conditions: Tests were conducted in 150 ml glass jars filled with 100 ml of unspecified medium. For first experimental setup, immobile *Daphnids* were counted after 24 hours after addition of the chemical, in a different experimental setup, immobile specimens were counted at 48 hours after addition of the chemical.

Replicates per concentration: 2

Organisms per replicate: 10

Feeding: None

Parameters measured: Immobility at 24 and 48 hours, water temperature. Immobility data were evaluated using probit analysis

Test concentrations: Preliminary experiment: 0.01% - 100% of unspecified test substance
0, 0.0115, 0.018, 0.021, 0.028, 0.032 mg Roundup®/L

Analytical determination of test concentrations: Not measured

5. Environmental conditions:

Test medium: Unknown origin:
26.5 µg/L Al
1 mg/L Cl
76 µg/L Fe
1.4 mg/L SO_4
6.9 mg/L Na

Temperature: 20 ± 2.0 °C

Photoperiod: 16 h light

Light intensity Not stated

pH: 8.1 (stock medium)

Oxygen saturation 2.8 mg/L

Conductivity: 161.2 µS/cm

Hardness: Not reported, but conductivity indicates low to moderate hardness

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable** (study carried out according to a method which is not acceptable, documentation not convincing for an expert judgement).

Comment:

- Validity criteria of OECD 202 not fulfilled.
- Clear description of the test substance missing; not clear which formulation (e.g. concentrated lawn or garden, agricultural formulation) was tested.
- Identical abiotic water parameters indicate that measurements were only performed on stock medium prior to the addition of test substance.

- Many relevant experimental details not stated, e.g. age of *Daphnids*, acclimatisation, test medium, pH values at start and end of exposure.
- No information about maintenance of exposure concentration provided
- Not clear whether endpoints reported refer to mg a.s./L, mg formulation/L or mg a.e./L.

2. Relevance of study: **Not relevant**

Comment: Due to substantial methodological flaws, study is not considered to be relevant.

3. Klimisch code: A clear description or specification of the test substance and material and methods applied is missing; no content of active substance is stated. In addition, oxygen content is too low and exhibits additional stress to specimens. This renders the study 'not reliable', resulting in a **Klimisch rating of 3 and not relevant for risk assessment.**

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Author(s)	Year	Study title
Sobrero, M. C., Rimoldi, F., Ronco, A.E.	2007	Effects of the glyphosate active ingredient and a formulation on *Lemna gibba* L. at different exposure levels and assessment end-points Bulletin of Environmental Contamination and Toxicology Volume: 79 Issue: 5 Pages: 537-543 DOI: 10.1007/s00128-007-9277-5 ISSN: 0007-4861 (print)

Abstract[64]

The use of formulations of the herbicide glyphosate in transgenic crops of the Pampa's plains of Argentina has extensively increased, though there is scarce information of its impact on non-target vascular plants from agro-ecosystem related surface waters. The sensitivity of a local clone of the macrophyte *Lemna gibba* L. to glyphosate active principle and Roundup Max formulation was studied in standardized laboratory conditions. Phytotoxic effects, considering the aquatic route, at a concentration range of glyphosate between 0.5 and 80 mg L^{-1} as active ingredient during 10 days of exposure were assessed on plant population growth, frond growth, shape and number, total chlorophyll content and colony architecture. Exposure to 1 mg L^{-1} of glyphosate (an expected environmental concentration) affects all the studied assessment endpoints, except for population growth and chlorophyll content. Equivalent concentrations of this herbicide as the active ingredient or RoundupMax indicate higher phytotoxicity of the formulation. Exposed plants at concentrations of herbicide between 1 and 7.5 mg L^{-1} exhibit after two days a recovery of the multiplication rate. Frond aggregation and longer stipe was detected between 1 and 15 mg L^{-1} of glyphosate, determining more open colony architecture. At higher concentrations of the herbicide fronds break-up. Comparisons with literature data indicate a higher sensitivity of the *L. gibba* local clone with respect to *L. minor* and algal species, and also a similar response to the herbicide in field experiments with the same species.

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate acid (technical grade)
RoundupMax®

Active substance(s): Glyphosate acid

Adjuvant/surfactant: Not stated

Description: none

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: Glyphosate acid (technical grade): 95% w/w
RoundupMax®: 70.7% w/w, a.s. as acid (a.e.).

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Lemna gibba*, local clone

Source: Field collected in El Pescado stream, Buenos Aires Province, Argentina

[64] Quoted from article

Growth stage at treatment:	Six fronds
Holding conditions prior to test:	Stock cultures were maintained under standardized growth conditions using a sterile nutrient solution with pH = 6.5, 250 µM NH_4NO_3, 220 µM $CaCl_2$, 406 µM $MgSO_4$, 30 µM K_2HPO_4, 500 µM $NaHCO_3$, 18µM EDTA-$FeCl_3$, 17.8 µM H_3BO_3, 1.8 µM $MnCl_2$, 0.08 µM $CoCl_2$, 0.16 µM $ZnSO_4$, 0.08 µM $CuSO_4$ and 1.4 µM Na_2MoO_4 at 24±2 °C under 16:8 light:dark cycle with 80 µM/m² s cool white fluorescent light. Plants were acclimatized for 1 month.

4. Test system:

Study type:	Static
Guideline:	None; study design comparable to OECD 221
GLP:	No
Guideline deviations:	- Stock medium similar to Steinberg medium (ISO 20079, see OECD 221), but additionally contains high amount of ammonium nitrate (fertilizer) - 1 mL of fivefold nutrient solution was added every 2-3 days. - Temperature for stock culture was above the range of 4 - 10 °C.
Duration of study:	10 days
Test conditions:	Tests were conducted in 500 ml jars filled with 2300 ml sterile nutrient solution. Every 2-3 days, a five-fold nutrient solution was added to the replicates to ensure exponential growth.
Treatments:	Glyphosate acid (technical grade), RoundupMax® and untreated control.
Test concentrations:	7 test concentrations: 0.5, 1.0, 7.5, 15, 25, 60 and 80 mg/L (nominal)
Replicates per concentration:	4
Fronds per replicate:	Not stated
Parameters measured:	pH, growth rate after 2, 5, 7 and 10 days, frond growth, frond number/colony , total chlorophyll content (TCC) and root length after 7 and 10 days. Data were evaluated using factorial ANOVA with Tukey as post-hoc test, effect concentrations were calculated by non-parametric linear interpolation.
Analytical determination of test concentrations:	At start and end of exposure by HPLC-UV

5. Environmental conditions:

Test medium:	Sterile nutrient solution (see above).
Temperature:	24±2 °C
Photoperiod:	16 h light
pH:	6.5 – 7.8
Conductivity:	Not stated
Hardness:	Not stated
Alkalinity:	Not stated
Experimental period:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**.

Comment:

- A number of deviations from the common OECD guideline lowered the reliability of the study.
- Most critical is the fact that endpoints are only based on nominal concentrations although measured concentrations deviated more than 20% from nominal concentrations.
- Separation factor of test concentration is higher than recommended.
- Though the deviations from the guideline the study is well-documented and statistics are sound.

2. Relevance of study: **Relevant with restrictions**

Comment: Effect of RoundupMax® is considered higher than for glyphosate technical, suggesting that this increase in toxicity is due to a surfactant, but it is not specified which surfactants/adjuvants RoundupMax® contains. The effect concentrations (EC_{50}) are within the range of values previously determined using GLP studies and provides confirmatory information for the endpoints generated the ecological assessment of glyphosate The formulation that was tested is MON 14420, a dry formulation and not MON 52276.

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
Sobrero, C., Martin, M.L., Ronco, A.	2007	Fitotoxicidad del herbicida Roundup® Max sobre la especie no blanco *Lemna gibba* en estudios de campo y laboratorio. Hidrobiológica Volume: 17 Supplement no. 1 Pages: 31-39 DOI: - ISSN: 0188-8897 (print)

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Abstract[65]

The use of the herbicide formulate Roundup® in transgenic crops of the Pampa's plains of Argentina has extensively increased, though there is scarce information on its impact on non-target vascular plants from agro-ecosystem related surface waters. Within this frame, the sensitivity of the macrophyte *Lemna gibba* L. to Roundup® Max was studied in standardized laboratory conditions and field experiments. In the laboratory, phytotoxic effects were assessed on the growth rate (GR) and total chlorophyll content (TCC). The GR was affected at 1 mg/L and TCC at 7.5 mg/L as active ingredient. Effects varied along testing time: toxicity was higher at low concentrations at the beginning, but diminishing during exposure, while at higher concentrations toxicity increments during testing. Also, a reduction of frond and root growth was detected. Field experiments with caged plants placed close to field crops showed an inhibition of 35.5% in biomass production (dry weight) and 75.5% in TCC when a dose of 1 kg/ha was applied. Although, in a second dose, only an inhibition effect on TCC of 53.9% was detected. The assessment of the herbicide drift did not show an influence of this exposure route on the plants for the studied scenery. The lowest glyphosate concentration producing an effect in the laboratory is in agreement with detected levels of the herbicide in the water body associated with effects in field experiments.

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (as Roundup®Max)
Active substance(s):	Glyphosate acid (74.4%)
Adjuvant/surfactant:	POEA (up to 15%) and further not specified coadjuvants
Description:	None
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	74.4%

2. Vehicle and/or positive control: Nutrient solution, pH adjusted to 6.5 prior to exposure

3. Test organism:

Species:	*Lemna gibba*
Source:	A local clone isolated from plant samples collected at the Pescado creek, Buenos Aires, Argentina.
Crop growth stage at treatment:	Not applicable. Plant cultures were maintained under growth experimental conditions for one month prior to the initiation of the study.

4. Test system:

[65] Quoted from article

Study type: Toxicity bioassay

Guideline: None.

GLP: No

Guideline deviations: Not applicable

Duration of study: 10 days

Test conditions: Study was conducted at the Environmental Research Centre (Centro de Investigaciones del Medio Ambiente, CIMA) of the Universidad de la Plata, Argentina.

The toxicity assay was conducted under controlled laboratory conditions (24 ± 2 °C, 16 h light period, 80 µM/cm²/s mean radiation density) in 300 mL nutrient medium without renewal (250 µM NH_4NO_3, 220 µM $CaCl_2$, 406 µM $MgSO_4$, 30 µM K_2HPO_4, 500 µM $NaHCO_3$, 1.8 µM EDTA-$FeCl_3$, 17.8 µM H_3BO_3, 1.8 µM $MnCl_2$, 0.08 µM $CoCl_2$, 0.16 µM $ZnSO_4$, 0.08 µM $CuSO_4$, 1.4 µM Na_2MoO_4, pH 6.5). In order to maintain exponential growth conditions during the 10-day exposure period, nutrients were added every 3-4 days (1 mL 5x solution). Plants were exposed to the herbicide as solution in nutrient medium.

Treatments: 7 test concentrations, untreated (negative) control

Replicates per concentration: At least 4 per concentration and negative control

Plot size per treatment: Not applicable

Parameters measured: Population growth curves (number of fronds over time), growth rate (percent inhibition over time), total chlorophyll content and morphological changes.

Growth rate (GR) was calculated as follows: GR = 1000*(log Ft-log Fo/t (Fo: number of fronds at Day 0; Ft: number of fronds at Day t of exposure).

Total chlorophyll content (TCT) was estimated in N,N-dimethylformamide extracts from fresh biomass (0.05 g biomass, 4 mL solvent) by spectrophotometrical measurements 647 and 664 nm). However, only qualitative results (photography) were presented.

Results are statistical comparisons to untreated controls.

Test concentrations: 0.5, 1, 7.5, 15, 25, 60 and 80 mg/mL of active ingredient

Analytical determination of test concentrations: Glyphosate concentration (mg a.s./L) was verified after 10 days of exposure in the nutrient media containing a nominal concentration of 1 and 25 mg a.s./L. Sample pre-treatment and herbicide quantification was conducted according to Peruzzo *et al.* (*Memorias Conferencia Internacional Usos del Agua, Agua 2003*, Cartagena de Indias, Colombia, p. 35-42). Glyphosate concentration was determined by HPLC following derivatization with FMOC-Cl (9-Fluorenylmethyl chloroformate) and borate buffer.

5. Environmental conditions:

Soil at study site: Not applicable

pH: 6.5 at the beginning and 7.8 at the end of the 10 day-exposure period (median ± SD: 7.7 ± 0.13)

Organic matter: Not applicable

Cation exchange capacity:	Not applicable
Soil textural fractions:	Not applicable
Weather conditions:	Not applicable
Experimental period:	Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Documentation insufficient for assessment
- Limited information on test item
- Laboratory study without positive control
- Sufficient information on experimental design (test organism and test system including analytical determination of test concentrations) and statistical analysis. However, results are not or only poorly and not adequately presented.
- In particular, no data on analytical results are given; results from growth parameters are not tabulated but presented as graphs; no quantitative results from chlorophyll content determination are presented; treatment-related morphological changes of the test organism are described and discussed but not properly presented.

2. Relevance of study: **Not relevant**

Comment: The study addresses toxic effects on a non-target organism. However, the insufficient documentation of the study results does not allow drawing reliable conclusions on the phytotoxic effects of the test item.

Additionally the formulation tested is not the formulation under evaluation which significantly impacts the relevance of these study results. A *Lemna gibba* study for MON 52276 is summarized in section 10.8.2.1

3. Klimisch code: **Klimisch rating of 3 and not relevant for risk assessment.**

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Effects on aquatic plants including algae

Author(s)	Year	Study title
Sánchez, D., Graça, M.A., Canhoto, J.	2007	Testing the use of the water milfoil (*Myriophyllum spicatum* L.) in laboratory toxicity assays. Bulleting of Environmental Contamination and Toxicology. Volume: 78 Pages: 421-426 DOI: 10.1007/s00128-007-9131-9 ISSN: 0007-4861 (print); 1432-0800 (web)

Abstract[66]

Tests aiming to determine the toxic properties of compounds discharged into aquatic systems have relied more on fish or invertebrates than on primary producers and among a number of producers; algae are the most popular test organisms. Macrophytes are important ecological elements in freshwaters and are therefore potentially key organisms for use in toxicity testing of compounds suspected of acting in primary producers. The most common macrophyte used in toxicity testing is *Lemna* sp., but as a floating plant, it has the limitation of being exposed to toxic compounds only through its lower leaf surface, including roots and rhizoids. Therefore, it is questionable whether tests with *Lemna* may accurately predict potential effects on submersed and exposed plant species, which have different routs of exposure and morphology. Few other submersed macrophytes have been tested, notably *Myriophyllum*. In the Iberian peninsula *M. spicatum* is the most common species within its genus and has been presented as a good bioaccumulator of heavy metals (Wang et al. 1996) and as being sensitive to several toxicants (e.g. Hanson et al. 2003). The aim of this study was to assess the potential of *M. spicatum* as a testing organism in laboratory assays, by obtaining axenic cultures of this plant and exposing them to several reference compounds to determine the sensitive endpoints.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup®, calcium, iron, copper sulphates and a mining effluent.
Active substance(s):	Glyphosate isopropylamine-salt (glyphosate-IPA)
Adjuvant / Surfactant:	Polyoxyethylene amine (POEA) is presumed to be present in the formulation and confirmed with a reference to previous study (Tsui & Chui 2003[67]).
Description:	The study addresses compounds related to mining and industrial pollution. Among these compounds glyphosate represented a non-selective post-emergent herbicide.
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	Not stated
Stock solution:	None

2. Vehicle and/or positive control:

3. Test organism:

[66] Quoted from article
[67] Tsui & Chu (2003) Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52: 1189-1197.

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Species:	*Myriophyllum spicatum* L. (Aquatic macrophyte)
Age of test organisms at study initiation:	Unknown
Source:	Field-collected from Carreiras River in the Guadiana basin, South Portugal.
Holding conditions prior to test:	Plants were rinsed in deionized water and nodal segments were disinfected in a 3% (w/v) sodium or calcium hypochlorite solution containing 0.01% Tween-20 for 20 minutes for 3 consecutive days. Kept in the dark between sterilization periods in Andrews modified medium with 3% sucrose (ASTM 2000[68]).
Acclimatisation:	Not specified.

4. Test system:

Study type:	Static, renewal (weekly)
Guideline:	American Society for Testing and Materials E1913-97 guide for *M. sibiricum* (2000) to obtain axenic cultures from field-collected stems.
GLP:	No
Guideline deviations:	Modified by using test species *M. spicatum*
Duration of study:	21 days
Test conditions:	3-cm apical shoots cut from plants were inoculated into 200 mL of sterile Andrew media containing the test item. The media was changed weekly and parameters were measured after 21 days.
Treatments:	5 substances with Roundup® as one of it in 9 test concentrations and 1 control.
Test concentrations:	Glyphosate concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 20, 40, 80 mg/L.
Replicates per treatment:	5
Parameters measured:	Fresh weight, shoot length, node number, root length.
Analytical determination of test concentrations:	Not stated
Validity criteria:	Not stated (might comply with criteria in the referred guideline ASTM 200)

5. Environmental conditions:

Test medium:	Andrews modified medium
Temperature:	25°C
Photoperiod:	14:10 hours light/dark phase
Light intensity	96 µm m^{-2} s^{-1}
Agitation:	Constant 100 rpm

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable.**

[68] American Society for Testing and Materials (2000) E 1913-97. Standard guide for conducting static, axenic, 14-day phytotoxicity tests in test tubes with the submersed aquatic macrophytes *Myriophyllum sibiricum Komarov*.

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Comment:
- It is not specified whether test concentrations refer to amount of formulated product or the active ingredient.
- No analytical verification of actual concentrations.
- Control growth under optimal growing conditions achieved only a 26% increase in length after 21 days. This would not meet the expectation of the AMRAP test guideline method which expects to see at least 50% increase within 7 days. This low growth rate seriously questions the validity of this study. From this perspective, the results in Sanchez should be considered with caution
- They were a low number of plants with high replicate variability and this was indicated in conclusions and consequently design not as robust as current AMRAP standards.
- There is no justification for extending test to 21 days. Growth media supplemented with sucrose - therefore does not reflect field situation.

2. Relevance of study: **Not relevant**

Comment: Testing a glyphosate-based herbicide plays only a minor role in this study so that authors neglected to specify important details about the herbicide. The study lacks information which would be crucial for an ecotoxicological evaluation. While the experimental approach is comparable to the AMRAP guidelines it simply does not specify source, purity, amount of active ingredient, actual test concentrations.
It is not clear what product was tested and whether concentrations refer to formulated product or active ingredient resulting in a Klimisch rating of not relevant.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

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Author(s)	Year	Study title
Turgut, C., Fomin, A.	2002	Sensitivity of the rooted macrophyte *Myriophyllum aquaticum* (Vell.) Verdcourt to seventeen pesticides determined on the basis of EC_{50}. Volume: 69 Issue: 4 Pages: 601-608 DOI: 10.1007/s00128-002-0103-9 ISSN: 0007-4861

Abstract[69]

Sensitivity of *Myriophyllum aquaticum* to 17 pesticides, amongst them a 360 EC glyphosate formulation, was investigated in a 14 day static toxicity test. Five replicates per treatment were exposed for 14 days to 7 or 8 pesticide concentrations. Plant length was established on alternate days, several endpoints such as chlorophyll a and chlorophyll b content, carotenoid and increases in shoot length, root number, total root length, fresh weight, side shoot number and side shoot length were measured at the end of exposure.

For the glyphosate 360 EC formulation, effective concentrations of the active substance (EC_{50}) was 0.222 mg a.s./L for chlorophyll a and b and carotenoid. The most sensitive endpoint was area under the growth curve (0.221 mg a.s./L). For increase in fresh weight, increase in shoot length and total root length, EC_{50} values of 1.999, 2.040 and 1.998 mg a.s./L were determined.

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate 360 EC formulation (specific formulation tested not provided)

Adjuvant/surfactant: Not stated/not determined

Description: Not stated

Source of test substance: German retailer, not further specified

Lot/Batch #: Not stated

Purity: 35.6%

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Myriophyllum aquaticum*

Growth stage of test organisms at study initiation: Not applicable

Source: Not stated

Holding conditions prior to test: Stock cultures were maintained by cutting 1 cm long stems into segments and transferring into culture vessels. After 4-6 weeks, 3 cm long axillary buds were transferred into 200 × 25 mm culture tubes containing 50 mL of sterile liquid growth medium.

4. Test system:

Study type: Static

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[69] Compiled by DKC because this journal does not provide abstracts in their publications.

Guideline: None; study design comparable to Maltby, L., *et al.* (2008): Aquatic Macrophyte Risk Assessment for Pesticides, SETAC AMRAP

GLP: No

Guideline deviations: Due to omissions in 'Materials and Methods' section, deviations to guideline cannot be determined.
No control data presented, therefore validity cannot be determined.

Duration of study: 14 d

Test conditions: Tests were conducted in 200 × 25 mm culture tubes filled with 50 ml solution. Test tubes were covered with sterile plain closures.

Treatments: 17 pesticide formulations, amongst them a Glyphosate 350 EC formulation and untreated controls.

Test concentrations: 7 or 8 test concentrations in each pesticide treatment, not further specified.

Replicates per concentration: 5

Organisms per replicate: Not stated

Parameters measured: Plant length was established on alternate days, chlorophyll a and chlorophyll b contents, and carotenoid were measured spectrophotometrically. Increases in shoot length, root number, total root length, fresh weight, side shoot number and side shoot length were measured by sight after 14 days of exposure. EC_{50} values were calculated using non-linear regression analysis.

Analytical determination of test concentrations: Not measured

5. Environmental conditions:

Test medium: Sterile liquid Hoagland growth medium supplemented with 3% sucrose and filled with 5 g Turface®

Temperature: About 25 °C (day)
18 – 20 °C (night)

Photoperiod: 16 h light

Light intensity: 120 – 180 µmol/m²/s

pH: Not stated

Oxygen saturation: Not stated

Conductivity: Not stated

Hardness: Not stated

Alkalinity: Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:
- Omissions in the methodical descriptions lowered the reliability of the study.
- Most critical is the fact that concentrations tested are not reported and composition of test substance is not specified

with regard to surfactants/adjuvants.

- Number of specimens per replicate is not reported.
- Source of *M. aquaticum* culture is not reported.
- No analytics reported/conducted.

2. Relevance of study: **Not relevant**

Comment: This non-GLP study may give some evidence for differences in sensitivity for different endpoints tested, the endpoint itself is considered not reliable. Most importantly, the particular glyphosate formulation tested was not provided which does not allow this study to be reliably used in an ecological assessment.

3. Klimisch code: **Klimisch rating of 3 and not aceeptable for risk assessment.**

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Author(s)	Year	Study title
Vera, M.S., Lagomarsino, L., Sylvester, M., Pérez, G.L., Rodríguez, P., Mugni, H., Sinistro, R., Ferraro, M., Bonetto, C., Zagarese, H., Pizarro, H.	2010	New evidences of Roundup® (glyphosate formulation) impact on the periphyton community and the water quality of freshwater ecosystems. Ecotoxicology Volume:19 Issue: 4 Pages: 710-721 DOI: 10.1007/s10646-009-0446-7 ISSN: 0963-9292 (print), 1573-3017 (online)

Abstract[70]

Argentina is the second largest world producer of soybeans (after the USA) and along with the increase in planted surface and production in the country, glyphosate consumption has grown in the same way. We investigated the effects of Roundup® (glyphosate formulation) on the periphyton colonization. The experiment was carried out over 42 days in ten outdoor mesocosms of different typology: "clear" waters with aquatic macrophytes and/or metaphyton and "turbid" waters with great occurrence of phytoplankton or suspended inorganic matter. The herbicide was added at 8 mg L^{-1} of the active ingredient (glyphosate) in five mesocosms while five were left as controls (without Roundup® addition). The estimate of the dissipation rate (*k*) of glyphosate showed a half-life value of 4.2 days. Total phosphorus significantly increased in treated mesocosms due to Roundup® degradation what favored eutrophication process. Roundup® produced a clear delay in periphytic colonization in treated mesocosms and values of the periphytic mass variables (dry weight, ash-free dry weight and chlorophyll *a*) were always higher in control mesocosms. Despite the mortality of algae, mainly diatoms, cyanobacteria was favored in treated mesocosms. It was observed that glyphosate produced a long term shift in the typology of mesocosms, "clear" turning to "turbid", which is consistent with the regional trend in shallow lakes in the Pampa plain of Argentina. Based on our findings it is clear that agricultural practices that involve the use of herbicides such as Roundup® affect non-target organisms and the water quality, modifying the structure and functionality of freshwater ecosystems.

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (Roundup ®) but particular formulation unknown.
Active substance(s):	Glyphosate IPA salt but loading unable to confirm
Adjuvant / Surfactant:	polyethoxylated tallowamine (POEA) stated in paper but not confirmed
Description:	none
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	Glyphosate: appears to be 480 g/L but cannot be confirmed by the information provided in the paper.
Stock solution:	Not stated

2. Vehicle and/or positive control: None

3. Test organism:

Species investigated:	Periphyton community
Source:	Not stated

[70] Quoted from article

Maturation and inoculation of organisms: Natural succession

Acclimatisation: Not applicable

4. Test system:

Study type: Mesocosm

Guideline: None

GLP: Not stated

Guideline deviations: -

Duration of study: April – June 2006

Test conditions: Mesocosm (1.2 m × 25 m²) were filled with well water and left to evolve naturally for 1 year, representing the typology of eutrophic shallow lakes in Argentine Pampean plain. At the beginning of experiment, artificial substrates (periphytometer) were introduced for periphyton colonisation. Following application of Roundup®, water samples were collected at six occasions using a Van-Dorn-style bottle.

Application: Not stated

Application devices: Not stated

Treatments: 5 treated and 5 untreated mesocosm ponds

Test concentrations: 8 mg glyphosate/L (nominal), 8.456 mg/L (initial mean measured)

Replicates per treatment: 5

Organisms per replicate: Periphyton community

Additional nutrient supply: None, not required

Parameters measured: Water samples collected 0, 3, 8, 14, 28 and 42 days after application were analysed for soluble reactive phosphorus, nitrate, nitrite, ammonium, Ca, Mg, Na, K, bicarbonate, sulphate, chloride,total phosphorus and for conductivity, pH and dissolved oxygen. Phytoplankton chlorophyll a content was monitored daily using an underwater turbidimeter, water transparence was measured at day 1.

Artificial periphyton substrates were collected after 8, 14, 28 and 42 days and stored in Lugol's solution. Counts of periphyton algae was conducted using an inverted microscope, live and dead diatom abundance was determined, algal class percentages, chlorophyll a concentration, dry weight, ash free dry weight and primary production.

Analytical determination of test concentrations: 0, 3,8, 14, 28 and 42 days after application by reversed-phase HPLC

Validity criteria: Not applicable

5. Environmental conditions:

Test medium: Matured well water

Temperature: Environmental conditions

Photoperiod: Not stated

Light intensity: Not stated

pH: 8.18 – 9.64

Dissolved oxygen: 5.5 – 11.5 mg/L

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Conductivity: 2.7±0.1 mS/cm
Nitrate: 1.8 – 12.3 mg N/L
Nitrite: 239 ± 107 µg N/L

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment:
- Even before application, huge differences between limnological properties of different mesocosm reported. This is an important point because there was no ecological parity within and between treatments seriously questioning the validity of the study.
- Formulation details not reported which is inappropriate to use in an ecotoxicological evaluation.

2. Relevance of study: **Not relevant**

Comment: The concentration of glyphosate used for the mesocosm exposure is substantially higher than what is to be expected to reach surface waters by drift, runoff and drainage events following application of products containing glyphosate according to the proposed good agricultural practices. This greatly diminishes the relevance of the study for an ecological risk assessment.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment**

Extended comments

While the authors suggest that the study results are more consistent with a direct toxicological effect of glyphosate than an indirect effect mediated by phosphorus enrichment, no direct comparison of glyphosate treatments with enhanced nutrient treatments was reported to conclusively demonstrate that effects observed in this paper are the result of the direct effect of glyphosate treatment.

The concentrations of the Roundup formulation tested in this study far exceed concentrations expected in the environment from the use of Roundup branded herbicides in glyphosate-resistant soy production.

In the paper, the authors refer to the increase in glyphosate use as a result of increased production of glyphosate-resistant soy. When used for weed control in soy, crop protection products have the potential to reach aquatic environments by three primary routes: drift, subsurface drainage, or surface runoff. Glyphosate exposure from subsurface drainage and surface runoff are comparatively low because glyphosate binds strongly to soil particles, and there is little leaching of glyphosate below the upper few centimeters of the soil. Therefore, drift during application is the primary route for glyphosate to reach aquatic environments.

The glyphosate concentration tested exceeds an extreme worst case concentration resulting from the direct over-spray of a very shallow water body. The tested concentration exceeds the FOCUS Tier 1 PECsw by approximately 80-times.

Most importantly, the concentrations of glyphosate IPA salt in the treatments in this study greatly exceed glyphosate concentrations that have been measured in surface water samples taken from streams and rivers that drain major agricultural areas where glyphosate tolerant crops have been produced for several years prior to the initiation of sampling (Scribner et al., 2007). These measured concentrations are from extensive water monitoring studies conducted in the United States by the U.S. Geological Survey (USGS), but may be considered as a starting point to assess potential concentrations in soy production areas in Argentina. For example, the concentrations tested in the Vera et al. study were approximately 1000-times higher than the maximum levels measured in flowing streams and approximately 20-times higher than in samples collected directly from water running off of a field on which glyphosate had been applied just a few days before the runoff event. Considering the total number of samples reported by Scribner et al.

(over 1200), the tested concentrations in the Perez et al. study are over 100 times higher then 99% of the analyzed samples and over 1000 times higher than 95% of the analyzed samples.
It is important to emphasize that Roundup branded products are not intended to be applied to surface water. In most agricultural uses, applications are made with ground equipment, which limits the amount of the formulation that will drift out of the field. Therefore, the effects observed in the work by Vera et al. occurred under conditions and concentrations that are not representative of Roundup branded product use situations.

References

Scribner, E.A., Battaglin, W.A., Gilliom, R.J., and Meyer, M.T. (2007) Concentrations of glyphosate, its degradation product, aminomethylphosphonic acid, and glufosinate in ground- and surface-water, rainfall, and soil samples collected in the United States, 2001-06. U.S. Geological Survey Scientific Investigations Report 2007-5122, 111 p.

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Effects on Bees

There is an extensive regulatory database assessing the acute contact and oral toxicity of glyphosate and glyphosate salts. The results from all of these studies demonstrate that glyphosate, glyphosate salts and MON 52276 have very low acute contact and oral toxicity to honeybees (section IIA Table 8.7.1 and IIIA Table 10.4-1). The 2001 EU glyphosate evaluation concluded that the hazard quotient values for intended uses of glyphosate are well below 50, indicating low acute toxicity for contact and oral routes of exposure according to the EPPO risk assessment scheme. Additionally, a bee brood study was recently performed for glyphosate following established methodology and demonstrated no chronic risk to bee brood at worst-case field exposure levels (overview and risk assessment provided in section IIIA10.4). This result from the bee brood study is consistent with the acute toxicity data and the results of independent chronic studies published in the literature. A brief overview of these chronic studies from the literature is provided below.

As far back as two decades ago, a field study was conducted to investigate the effects of aerial application on honey bee hives (Burgett and Fisher 1990). Bee hives and blooming vegetation in the immediate vicinity (1.5 acres) were treated at a high rate of approximately 5% of a Roundup formulation in 60 gal of water (6.8 kg a.e./ha). No acute or chronic effects were observed for adult honey bees or for brood production. These findings were further supported by conducting direct feeding trials in the field. No effects were observed as a result of the direct addition of Roundup to the hives. Additionally, a brood study performed in Australia found not impact on larval and adult bees after six days of exposure to 5 mg a.s./kg sucrose solution and concluded that glyphosate could be used safely around hives (Ferguson, 1987; Ferguson, 1988). Taking the weight of evidence from the results with acute and chronic studies together, honey bees are not affected by glyphosate or Roundup formulations in the field.

In this section we address a recent article that investigated the effect of glyphosate on honey bee gut histology.

References:

Burgett, M. and Fisher, G. 1990. A review of the Belizean honey bee industry: Final report prepared at the request of The Belize Honey Producers Federation. Department of Entomology, Oregon State University, Corvallis, Oregon.

Ferguson, F. 1987. Interim report. Long term effects of systemic pesticides on honey bees. The Australian Beekeeper. Pages: 49-53 (September issue).

Ferguson, F. 1988. Long term effects of systemic pesticides on honey bees. Bee keeping in the year 2000: Second Australian and International Beekeeping Congress, Surfers Paradise, Gold Coast, Queensland, Australia, July 21-26, 1988. Editor: John W. Rhodes. Pages: 137-141.

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Author(s)	Year	Study title
Gregorc, A., Ellis, J.D.	2011	Cell death localization *in situ* in laboratory reared honey bee (*Apis mellifera* L.) larvae treated with pesticides Pesticide Biochemistry and Physiology Volume: 99 Pages: 200-207

Abstract

In this study, cell death detected by DNA fragmentation labeling and phosphatidylserine (PS) localization was investigated in the honey bee (*Apis mellifera* L.) midgut, salivary glands and ovaries after treating larvae with different pesticides offered via an artificial diet. To do this, honey bee larvae reared in an incubator were exposed to one of nine pesticides: chlorpyrifos, imidacloprid, amitraz, fluvalinate, coumaphos, myclobutanil, chlorothalonil, glyphosate and simazine. Following this, larvae were fixed and prepared for immunohistologically detected cellular death using two TUNEL techniques for DNA fragmentation labelling and Annexin V to detect the localization of exposed PS specific *in situ* binding to apoptotic cells. Untreated larvae experienced ~10% midgut apoptotic cell death under controlled conditions. All applied pesticides triggered an increase in apoptosis in treated compared to untreated larvae. The level of cell death in the midgut of simazine-treated larvae was highest at 77% mortality and statistically similar to the level of cell death for chlorpyrifos (65%), imidacloprid (61%), myclobutanil (69%), and glyphosate (69%) treated larvae. Larvae exposed to fluvalinate had the lowest midgut columnar apoptotic cell death (30%) of any pesticide-treated larvae. Indications of elevated apoptotic cell death in salivary glands and ovaries after pesticide application were detected. Annexin V localization, indicative of apoptotic cell deletion, had an extensive distribution in the midgut, salivary glands and ovaries of pesticide-treated larvae. The data suggest that the tested pesticides induced apoptosis in tissues of honey bee larvae at the tested concentrations. Cell death localization as a tool for a monitoring the subclinical and sub-lethal effects of external influences on honey bee larval tissues is discussed.

MATERIALS AND METHODS

1. Test material:

Test items (active substances): Chlorpyrifos, Imidacloprid, Amitraz, Fluvalinate, Coumaphos, Myclobutanil, Chlorothalonil, Glyphosate, Simazine

Source: Chem Service, West Chester, PA, USA

Description: Not reported

Lot/Batch #: Not reported

Purity: Not reported

2. Vehicle and/or negative control: Yes

Vehicle control: Acetone (diluent for each test item). **Glyphosate is not soluble in acetone**.

Negative control: Untreated diet

3. Test organism:

Species: *Apis mellifera* L. (Honey bee)

Source: University of Florida Honey Bee Research and Extension Laboratory, Department of Entomology and Nematology,

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	Gainesville, FL, US
Age of test animals at study initiation:	60 h
Acclimatisation:	2 days
Diet/Food:	50% royal jelly, 6% D-glucose, 6% D-fructose, 37% double distilled water, 1% yeast extract (pre-warmed to 35 °C); amount ranged from 20-50 µL per larvae and day depending on the larva's age.
Housing:	96- or 48-well plate depending on the larva's size.
Environmental conditions:	Temperature: 35 °C Relative humidity: 96%

4. Test methods:

Guideline:	None
Duration of study:	4 days
Bees per group:	12
Test concentrations in diet:	Chlorpyrifos: 1.6 ppm Imidacloprid, amitraz, myclobutanil, chlorothalonil, glyphosate and simazine: 400 ppm Fluvalinate: 200 ppm Coumaphos: 100 ppm Controls: untreated diet and diet containing acetone
Treatment:	Prior to administration to the larval diet, each pesticide was diluted individually in acetone solvent. Larvae received diet/pesticide mixtures for 4 consecutive days, beginning the second day the larvae were in the laboratory.
Sacrifice:	All larvae were sampled on day 6, 24 h after the last application of pesticide. Sampled larvae were fixed in 10% formalin for 24 h, dehydrated in a series of alcohols and xylene, and finally embedded in paraffin wax as described by Gregorc and Bowen [9]. Sections of 5 µm were cut on a Microtome, floated on distilled water at 40 °C, collected on cleaned slides, and kept in a drying oven at 60 °C for ca. 4 h. Slides then were stored at room temperature until later analyses.

5. Observations/analyses:

Immunohistologically detected cellular death:	**DeadEnd colorimetric TUNEL system** After applying proteinase K, the larval sections were incubated with the terminal deoxynucleotidyl transferase (TdT) reaction mixture and then with a horseradish peroxidise-labelled streptavidin solution. Diaminobenzidine (DAB) substrate was applied onto the tissue sections to develop a brown reaction product. The sections were counterstained with Mayer's hematoxylin. Negative control was achieved by substituting the deoxynucleotidyl transferase (TdT) enzyme with PBS. **In situ cell death detection kit, AP (ISCDDK)** Dewaxed and rehydrated tissue sections were incubated with proteinase K. Labelling was conducted by covering the tissue section with a TUNEL reaction mixture composed of TdT from calf Thymus. TdT enzymes with fluorescein were detected

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using "converter-AP" consisting of anti-fluorescein antibodies from sheep, conjugated with alkaline phosphatase. The substrate solution was obtained using a Vector® Red Alkaline Phosphatase Substrate Kit. Sections were incubated with the substrate (AP) and washed in tap water for 5 min. Counterstaining was accomplished by transferring the sections into Mayer's hematoxylin and then rinsing the sections under running tap water. As a negative control, a subgroup was labelled with terminal transferase, rather than TUNEL reaction mixture.

Immunohistological Annexin V localization: Rabbit antibodies polyclonal to Annexin V (2 μg/mL in PBS with 1% BSA) were incubated overnight at 4 °C, afterwards the sections were covered with biotinylated universal secondary antibodies for 30 min. Alkaline 486hosphatise reagent also was applied for 30 min. The substrate solution was obtained using a Vector® Red Alkaline Phosphatase Substrate Kit. Sections were incubated with the substrate (AP) and washed in tap water for 5 min. Counterstaining was accomplished by transferring the sections into Mayer's hematoxylin and then rinsing the sections under running tap water.

Quantification and statistics: Tunnel labelled tissue slides were used for quantification of cell type and apoptosis using two commercial kits (DeadEnd and ISDDK) with two different staining techniques. For each treated group of larvae, approximately 300 total cells from at least 3 different larvae on different slides were counted in random fields within the tissue. The results were expressed as the proportion of cells counted that gave positive staining. To confirm reproducibility, 25% of slides were chosen randomly and scored twice.

The proportion of cells that gave positive staining was analysed by treatment (9 pesticides and 2 controls) with a one way ANOVA for both staining techniques. In addition, two way ANOVA was used to test the effects of technique, overall treatment and the interaction of treatment x technique on the proportion of cells with positive staining. Prior to analyses, the proportion data were transformed with an α sin $\sqrt{x}$ transformation. The untransformed means are reported. Where necessary, Student´s t-test was used to compare means, accepting differences at $p \leq 0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

The observed effect of feeding glyphosate at these high concentrations to honeybees could be the results of a pH effect. Glyphosate at high concentration in aqueous solutions can results in very low pH.

Comment: **Not relevant**

2. Relevance of study: This was proof of concept study that tested on doses diminishing its relevance. No justification was provided for a 6 day study exposing bees to 400 ppm glyphosate other than this is the highest residue level found in hives from a list pesticide

	that did not include glyphosate. Honey bees will not have a sustained exposure to these levels of glyphosate in the field. Exposure information in section IIA 8.7.3/01 demonstrates that levels in pollen and nectar rapidly decline with a DT_{50} of approximately 1 day and 1.5 days in nectar and pollen, respectively. A brood study, summarized in section IIA 8.7.3/02, demonstrates no impact on honeybee survival and brood development.
3. Klimisch code:	**3 and not acceptable for risk assessment**

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Author(s)	Year	Study title
Gregorc A, Evans J. D, Scharf, M., Ellis, J.D.	2012	Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and *Varroa* mites (*Varroa destructor*) Journal: Journal of Insect Physiology Volume: accepted manuscript (electronic publication ahead of print) Issue: - Pages: - DOI: 10.1016/j.jinsphys.2012.03.015 ISSN: 0022-1910

Abstract[71]

Honey bee (*Apis mellifera*) larvae reared in vitro were exposed to one of nine pesticides and/or were challenged with the parasitic mite, *Varroa destructor*. Total RNA was extracted from individual larvae and first strand cDNAs were generated. Gene-expression changes in larvae were measured using quantitative PCR (qPCR) targeting transcripts for pathogens and genes involved in physiological processes, bee health, immunity, and/or xenobiotic detoxification. Transcript levels for Peptidoglycan Recognition Protein (PGRPSC), a pathogen recognition gene, increased in larvae exposed to Varroa mites (P<0.001) and were not changed in pesticide treated larvae. As expected, Varroa-parasitized brood had higher transcripts of Deformed Wing Virus than did control larvae (P<0.001). *Varroa* parasitism, arguably coupled with virus infection, resulted in significantly higher transcript abundances for the antimicrobial peptides abaecin, hymenoptaecin, and defensin1. Transcript levels for Prophenoloxidase-activating enzyme (PPOact), an immune end product, were elevated in larvae treated with myclobutanil and chlorothalonil (both are fungicides)(P<0.001). Transcript levels for Hexameric storage protein (Hsp70) were significantly upregulated in imidacloprid, fluvalinate, coumaphos, myclobutanil, and amitraz treated larvae. Definitive impacts of pesticides and Varroa parasitism on honey bee larval gene expression were demonstrated. Interactions between larval treatments and gene expression for the targeted genes are discussed.

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MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate but no details provided whether acid or salt or vendor

Active substance(s):

Adjuvant: Not stated

Description: Not stated

Source of test substance: Not stated

Lot/Batch #: Not stated

Purity: Not stated

Stock solution: Not stated

2. Vehicle and/or positive control: **Acetone – but glyphosate is not soluble in acetone**

3. Test organism:

Species: female larvae of *Apis mellifera*

[71] Quoted from article

Cultivar: Not stated

Source: Langstroth-style production colony located in Gainesville, Florida (USA)

Age of test organisms at study initiation: 156 h

Holding conditions prior to test: Rearing: Between 24 - 108 h after oviposition eggs and developing larvae stayed in the metal queen excluder cage (~10 x 10 x 3 cm) and were fed by bee workers, the cage were located on the comb, after 108 h larvae were transported into the laboratory and grafted were grafted into sterile, 96-well tissue culture plates (well volume = 0.32 mL, Fisher Scientific, Pittsburgh, PA, USA), wells are pre-warmed to 35 °C,

Acclimatisation: Not stated

4. Test system:

Study type: Laboratory

Guideline: None

GLP: Not stated

Guideline deviations: -

Duration of study: between 154 h and 180 h after oviposition (diet with pesticides) + 48 h (infection with mites)

Test conditions:

- Experiments were conducted at the Honey Bee Research and Extension Laboratory at the University of Florida's Department of Entomology and Nematology (Gainesville, FL, USA) and at the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) Bee Research Laboratory in Beltsville, MD, USA.
- Larvae reared in an incubator
- Feeding: 30 µL on hour 156, 40 µL on hour 180, and 50 µL on hour 204
- at hour 204 larvae were transferred to 48-well plate (13 x 17 mm)
- later than 180 h, larvae were infected with *Varroa* mite
- 12 Larvae from each group are allowed to reach the pre-pupal stage once they defecated
- Mites (phoretic stage) are obtained from 300 infected worker bees transferred in a 0.5 L jar, coating the bees with approximately 30g of 10x confectionary sugar, shaking the jar lightly for 30 seconds, and collecting mites falling from the screened jar with a sieve

Water volume: -

Treatments:

1) 1 treatment with glyphosate (+ 8 additional pesticides treatments) 2) 1 control group with a mixture of a diet with acetone, 3) 1 control with untreated diet	a) After first defecation: individuals without and 12 indiv. with 2 adult mites of the genus *Varroa destructor*	– 8 indiv. per group for molecular analysis

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Replicates per concentration:	24
Individuals per replicate:	1
Parameters measured:	Transcript abundance Gene expression changes were measured for bee health immunity, xenobiotic detoxification (RNA extraction with Triazol reagent reagent (Invitrogen, Carlsbad, California, USA), cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen))
Test concentrations:	200 ppm glyphosate diluted in acetone solvent
Application / device:	Pesticides are given via diet (see above)
Feeding during experiments:	• Diet composition: Diet: 50% royal jelly (Glory Bee Foods, Eugene, OR, USA), 6% D-glucose (Fischer Chemical, Fair Lawn, NJ, USA), 6% D-fructose (Fischer Chemical, Fair Lawn, NJ), 37% double distilled water, and 1% yeast extract (Bacto™, Sparks, MD, USA) by volume (Aupinel et al., 2005), pH 4.0 – 4.5, • Feeding: 20 µL diet at hours 108 and 132, 30 µL on hour 156, 40 µL on hour 180, and 50 µL on hour 204
Verification of dispersion:	Not stated
Validity criteria:	Not stated

5. Environmental conditions:

Test medium:	-
Temperature / relative humidity:	35°C / 96% RH
Photoperiod:	-
Lighting:	None
pH:	-

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- No information about physical stage of queen bee as recommended in the OECD guideline for worker bees.
- Data about time after oviposition in which the larvae were infected of mites are missing, it may at the very most be implied that this occurred at the same time as pesticide feeding started.
- The test was conducted only with a single test concentration, so that there could be no conclusion on dose-response-gene-expression
- Glyphosate was mixed with acetone as a solvent. It is well known that glyphosate is insoluble in acetone.
- Unknown source and form of the test substance.
- Methodology to quantify target gene expression was not quantitative. Isolated mRNA was converted to cDNA and this step in the procedure can result in varying efficiencies between reaction tubes leading to artifacts in gene expression comparisons. This can results from differences is quality of isolated mRNA and inherent variability of

specific primers. These investigators used random primers. This approach primes the reverse transcription at multiple origins along every RNA template producing more than one cDNA target per original mRNA target. Furthermore, the majority of cDNA synthesised from total RNA is ribosomal RNA (rRNA)-derived. This could create problems if the mRNA target of interest is present at low levels, as it may not be primed proportionately and its subsequent amplification may not be quantitative. This is particularly true if the assays ahs not been optimized and the paper provides no indication this methodology was optimized. Absolute quantification requires that the efficiency of the amplification reaction is the same in all samples and in the external quantified standards. Consequently, it is important that the efficiency of the PCR does not vary greatly due to minor differences between samples. Differences in reaction efficiency for the reverse transcriptase step can be addressed with inclusion of the appropriate internal controls, which were not included this analysis. Consequently, the differences in gene expression between control and glyphosate treatments may very likely be an artifact of the resulting from differential inter-assay variability for the reverse transcriptase reactions, the step prior to real time PCR analysis. These omissions question the reliability, relevance and reproducibility of the results for the real time quantitative PCR presented in this paper.

2. Relevance of study:

Comment: This was proof of concept study that tested on doses diminishing its relevance. Accurate quantification of mRNA levels requires that the efficiency of the amplification reaction is the same across all samples and in the external standard. This omission in the methodology challenges the reliability, relevance and reproducibility of the results for the real time quantitative PCR presented in this paper.

3. Klimisch code: **Klimisch rating 3 and not acceptable for risk assessment.**

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Effects on non-target terrestrial arthropods

Glyphosate-based herbicide formulations have been used as broad-spectrum herbicides for nearly 40 years. Many studies have been conducted to assess the environmental safety of the active substance and its formulations in the lab and the field. Screening tests have been performed on a number of other beneficial arthropods including beetles, mites, spiders, and wasps. These tests are designed to maximize exposure (maximum use rate, no interception, etc.). Large beneficial arthropods such as ground predators (spiders and beetles) are not affected by glyphosate formulations. Although most foliar dwelling species were not affected in the screening tests, several species were (e.g. parasitic wasp and predatory mite). The effects observed in these screening tests under extreme conditions were reduced or eliminated when more realistic exposure conditions were used.

Tier 1 laboratory tests are highly artificial tests where the pesticide is applied at the maximum use rate onto artificial substrates such as glass or synthetic soil and the organisms are forced to remain in contact with the pesticide film for several days. Depending on results of these screening tests, subsequent tests such as extended laboratory tests, semi-field and field tests may be needed. These "higher tier" tests incorporate more realistic environmental exposure conditions.

The effects of Roundup herbicide have been investigated in the screening level assay with 18 different beneficial predators and parasites (Hassan et al. 1988). Roundup was found to be harmless to thirteen species, slightly harmful to four species and moderately harmful to one species of carabid beetles. The authors did not believe that sufficient toxicity potential existed to warrant semi-field and field tests that were performed on some of the other compounds tested in the same program.

Moreover, a subsequent semi-field test with a similar glyphosate formulation was conducted on carabid beetles (Mead-Briggs, 1991). Results of that test indicated that even if beetles were directly over sprayed at the maximum use rate, no mortality was observed. The reason for the discrepancy between the laboratory and semi-field study results is not known but may be related to the highly artificial nature of the laboratory glass plate assays (e.g. potential stickiness of the formulation on the glass substrate).

Beneficial arthropod tests have been conducted on existing glyphosate formulations used in the European Union. The formulations at the maximum use rate caused no effects on beetles or spiders. Some effects were observed on parasitic wasps and predatory mites at the maximum use rate in the screening tests. As described earlier for Roundup, the effects were reduced or eliminated when real exposure conditions and substrates were included.

In addition, field studies have investigated the effects of glyphosate formulations on beneficial arthropods other than honey bees. No substance- or dose-related effects on mites or springtails were observed in a sandy soil in an Argentine semi-arid region up to 96 days after application of Roundup herbicide at rates up to 2.8 kg glyphosate/ha (Gomez & Sagardoy 1985). No effects were noted for the number of nematodes, mites, or springtails in the top 3 cm of soil 180 days after treatment with Roundup at 2 kg a.e./ha (Preston & Trofymow 1989).

Reduced populations of herbivorous insects and ground invertebrates were observed in a 4-5 year old clear-cut planted with spruce (Picea sp.) up to 3 years after treatment with Roundup herbicide (Santillo et al. 1989a; 1989b). During this 3 year study, the vegetation failed to recover completely, suggesting that the majority of effects on invertebrates were mainly due to habitat change. On the other hand, no changes in populations of predatory insects were observed in a clearcut located in Maine (United States) that was sprayed with 1.7 kg glyphosate/ha (Whitehouse and Brown 1993). The authors of the report concluded that insect communities would not be affected by the use of glyphosate applied to the base of trees in farm forestry.

The potential for effect of glyphosate on beneficial insects has also been investigated by studying indigenous carabid beetle populations after field application (Brust 1990). Both relative densities and movement of carabid beetles in and out of treated areas was monitored. No direct toxicity was observed in the field nor was there any repellency noted. Shifts in population densities were observed in the weeks following treatment, but these changes were more closely associated with changes in the vegetation of the plots rather than direct toxicity following the glyphosate treatment. The absence of direct toxic effects was confirmed in the laboratory. Other researchers concluded that the primary factors influencing the changes in carabid beetle and spider populations were deprivation of a particular species of suitable food and change in habitat (Asteraki et al. 1992). Several studies have shown that the application of glyphosate can increase populations of beneficial insects. In laboratory experiments to simulate treatment of cotton fields, numbers of the western bigeyed bug, *Geocoris pallens*, increased (Yokoyama and Pritchard 1984). However, these authors did not measure behavioral effects and cautioned that responses might vary under field conditions. No effects on the number of common butterfly species were observed when glyphosate was used to control trees, shrubs and blackberry in wire zones; but numbers of individuals did increase (Bramble et al. 1997).

Screening tests have been performed on a number of other beneficial arthropods including beetles, mites, spiders, and wasps. These tests are designed to maximize exposure (maximum use rate, no interception, etc.). Large beneficial arthropods such as ground predators (spiders and beetles) are not at risk from glyphosate formulations. Several foliar dwelling species (e.g. parasitic wasp, predatory mite) for several glyphosate formulations are potentially affected based on laboratory screening tests. However, under realistic exposure regimes, testing showed that it is unlikely that effects will be observed. Within treated areas, alteration of the vegetation following glyphosate treatment can result in substantial change in habitats over the short term, and, consequently, in some cases, insect populations.

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Author(s)	Year	Study title
Benamú, M.A., Schneider M.I., Sánchez N.E.	2010	Effects of the herbicide glyphosate on biological attributes of *Alpaida veniliae* (Araneae, Araneidae), in laboratory Journal: Chemosphere Volume: 78 Issue: 7 Pages: 871 - 876 DOI: 10.1016/j.chemosphere.2009.11.027 ISSN: 18791298

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Abstract[72]

In the past decades there has been increasing interest in the study of arthropod predators as effective potential natural enemies to be used in the biological control of agricultural pests. In Argentina, transgenic soybean crops (Round-up Ready, RR) are inhabit by many spider species, some of them in high abundance, being indicative of an import potential for pest predation. This crop is associated with the use of glyphosate, a broad-spectrum herbicide, with low environmental impact, even though since the 80's, several negative effects have been deeply documented on mammals, fishes, amphibians, snails, earthworms, insects, etc. Nowadays, the effects on arthropod physiology, behavior and life history traits as end-points in ecotoxicological evaluations are being recognized. In transgenic soybean crops of Buenos Aires province (Argentina), *Alpaida veniliae* (Araneae, Araneidae) is one of the most abundant orb web weaver spiders. The purpose of this study was to address the effects of glyphosate on some biological attributes of *A. veniliae*, in laboratory. Results of this study showed no lethal direct effects of Glifoglex ® on this spider, but it is the first report in literature about sublethal effects of this herbicide on a spider's biological attributes. Negative effects on prey consumption, web building, fecundity, fertility and developmental time of progeny were observed. Although sublethal effects have received less attention than direct lethal effects, they are relevant from an ecological point of view, since the reduction of the arthropod performance may create risks to arthropod biodiversity conservation in agroecosystems.

MATERIALS AND METHODS

1. Test material:

Test item:	Glifoglex 48 ® (Gleba SA, Buenos Aires, Argentina)
Active substance(s):	Glyphosate
Adjuvant:	Not stated
Description:	Not stated
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	48% Glyphosate
Stock solution:	192 mg a.i./L (= maximum field registered nominal concentration), acetone as solvent

2. Vehicle and/or positive control: None

3. Test organism:

Species:	*Alpaida veniliae* (adult females)
Cultivar:	None

[72] Quoted from article

Source: Collected from transgenic soybean crops located at Chivilcoy (35°01´S, 60°06´W) (Buenos Aires, Argentina), from January to March 2006.

Age of test organisms at study initiation: 15 d

Holding conditions prior to test: Adults were reared in 15 x 15 cm height and width glass jars (500 mL), and maintained in the laboratory to obtain egg sacs. Juveniles emerging from the egg sacs were followed to obtain a mass-rearing population. Juveniles and adults were fed *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) and *Musca domestica* L. (Diptera: Muscidae) adults, ‘‘ad libitum”. Laboratory conditions were 25 ± 2° C temperature, 75 ± 5% RH, and a photoperiod of 16:8 (L:D) h.
Reared females used for the test starved 1 week

Acclimatisation: Not stated

4. Test system:

Study type: Toxicity bioassay

Guideline: Not stated

GLP: Not stated

Guideline deviations: -

Duration of study: Lethal effects: 8 d
Sublethal effects: 20 d (development of ovaries were investigated after 15 d)

Test conditions: Lethal effects: Test vessel: 6 cm diameter Petri ® dishes, feeding: 4 d with treated food + 4 d with untreated food, rests of prey were daily removed
Sublethal effects: individuals were placed in wooden frames (15 x 10 x 5 cm) surrounded by glass to allow viewing web building

Application/ exposure route: Via treated food

Application devices: -

Water volume: -

Treatments: 1 (+ 1 control received food dipped in acetone)

Replicates per concentration: Lethal effects & web building: 60
Development of ovaries: 10
Fecundity & fertility: 5

Individuals per replicate: 1

Feeding during experiments: Daily with glyphosate treated *Musca domesica* L.

Parameters measured: Mortality, sublethal effects: feeding rate, number of web radius and spires, mating, development of ovaries, number of eggs per egg sac, malformation of eggs, hatching of spiderlings

Test concentrations: Food dipped in stock solution

Application / device: -

Verification of dispersion: Not stated

Validity criteria: Not stated

5. Environmental conditions:

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Test medium: None

Temperature: Presumably the same condition stated in the “Holding conditions prior to test”

Photoperiod: see “Temperature”

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- No information about the application rate, so it is impossible to compare the results with standard laboratory experiments for non target arthropods.
- Feeding in the control occurred with acetone dipped food, there was no approach without treated food
- Even though well-documented the method is not validated.
- It was not stated if the prey was alive. Probably it was, but the method of dipping the prey into the test solution seems not appropriate, because this method described by another author was conducted with eggs and not with living animals. It is unclear if the behaviour of test animals are influenced and likewise the prey consumption.
- The test substance was reported to be prepared using acetone; however glyphosate is not soluble in acetone and almost all organic solvents. There is no graphical presentation of the results of web building parameters and development of eggs, here only the mean with standard error is presented

2. Relevance of study: **Not relevant**

Comment:

- Little is known about the formulation that was tested other than it is reported to be 48% glyphosate and produced by Gleba SA, Buenos Aires, Argentina.
  No information about the application rate, so it is impossible to compare the results with standard laboratory experiments for non target arthropods.
- Although there are statistical significant effects after treatment, for comparable and reliable results it is essential to conduct an approach with treated food, food treated with the solvent and untreated food. Even the feeding seems not suitable. Although the study is well documented the study does not match basic scientific principles and is only vague with respect to material & methods in main parts.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment**

Author(s)	Year	Study title
Evans, S.C., Shaw, E.M., Rypstra, A.L.	2010	Exposure to a glyphosate-based herbicide affects agrobiont predatory arthropod behaviour and long-term survival. Ecotoxicology Volume: 19 Pages: 1249-1257 DOI: 10.1007/s10646-010-0509-9 ISSN: 0963-9292 (print); 1573-3017 (online)

Abstract[73]

Humans commonly apply chemicals to manage agroecosystems. If those chemicals influence the behaviour or survival of non-target arthropods, the food web could be altered in unintended ways. Glyphosate-based herbicides are among the most ubiquitous pesticides used around the world, yet little is known about if and how they might affect the success of terrestrial predatory arthropods in agroecosystems. In this study, we quantified the effects of a commercial formulation of a glyphosate-based herbicide on the activity of three predatory arthropod species that inhabit agricultural fields in the eastern United States. We also measured the survival of the most common species. We tested the reactions of the wolf spider, *Pardosa milvina*, to either direct application (topical) or contact with a treated substrate (residual). We quantified the reactions of a larger wolf spider, *Hogna helluo*, and a ground beetle, *Scarites quadriceps*, to a compound (topical plus residual) exposure. *Pardosa milvina* reduced locomotion time and distance under topical herbicide exposure, but increased speed and non-locomotory activity time on exposed substrate. Both *H. helluo* and *S. quadriceps* increased nonlocomotory activity time under compound herbicide exposure. Over a period of 60 days post-exposure, residually exposed *P. milvina* exhibited lower survivorship compared to topically exposed and control groups. Thus, exposure of terrestrial arthropods to glyphosate-based herbicides affects their behaviour and long-term survival. These results suggest that herbicides can affect arthropod community dynamics separate from their impact on the plant community and may influence biological control in agroecosystems.

MATERIALS AND METHODS

1. Test material:

Test item:	Buccaneer Plus©
Active substance(s):	Glyphosate (*N*-(phosphonomethyl)glycine) isopropylamine salt
Adjuvant:	59% other ingredients including what was speculated buy the authors to be polyethoxylated tallowamine (POEA)
Description:	Recommended concentration is 0.625-5% for use in agricultural fields.
Source of test substance:	Commercially purchased (USA)
Lot/Batch #:	Not stated
Purity:	41% (480 g/L)
Stock solution:	2.5% (12 g/L) glyphosate salt diluted with distilled water
2. Vehicle and/or positive control:	None
3. Test organism:	
Species:	*Pardosa milvina* (Araneae: Lycosidae) *Hogna helluo* (Araneae: Lycosidae)

[73] Quoted from article

	Scarites quadriceps (Coleoptera: Carabidae)
Source:	Field-collected from corn and soybean fields at the Miami University Ecology Research Center (Oxford, Ohio, USA)
Age of test organisms at study initiation:	Unknown, field-collected generation was directly used for trials.
Holding conditions prior to test:	Animals were kept individually in cylindrical plastic containers with a mixture of moist potting soil and peat moss in a 2-3 cm layer. All species were fed with *Acheta domesticus* crickets (2 x 0.6 cm length per week for *S. quadriceps* and *H. helluo* and 2 x 0.3 cm length per week for *P. milvina*) and water ad libitum. Animals were housed in climate chambers at 24°C on 13:11 light:dark cycle.
Acclimation:	*Pardosa milvina* was food-deprived for 5 days prior to trials while *S. quadriceps* and *H. helluo* were fed 48 hours prior to trials. At beginning of behavioural assays acclimation periods were allowed to subjects of 15 minutes and of 10 minutes for *P. milvina* and for the two other species, respectively.

4. Test system:

Study type:	Extended laboratory
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	Behavioural assay of 40-45 minutes (incl. 30 min. observation) Survival of *P. milvina* over 60 days after exposure
Test conditions:	The spider *P. milvina* was observed for behavioural alterations in a dual-choice residual exposure in which distilled water and glyphosate were sprayed in two semicircle areas. After an acclimation period in the treated arena, animals were observed for 30 minutes. All 3 test species were observed in a topical exposure experiment. Here distilled water or glyphosate were applied directly onto the animals and subsequently observed for behavioral changes for 30 minutes. Each individual was observed twice to analyse the impact of handling in addition. Survival of tested *P. milvina* was recorded for a period 60 days after exposure and behavioural observation.
Water volume:	93.54 – 374.16 L/ha
Treatments:	1 glyphosate treatment and 1 distilled water control
Replicates per treatment:	*P. milvina* (residual exposure): 30 *P. milvina* (topical exposure): 30 for glyphosate and 15 for water control *H. helluo* (topical exposure): 12 per treatment *S. quadriceps* (topical exposure): 11 per treatment
Individuals per replicate:	1
Arena size per treatment:	*P. milvina:* 20 cm in diameter with 8 cm walls. *H. helluo* and *S. quadriceps*: 24 cm in diameter with 10 cm walls

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Parameters measured: Time spent in regions, distance travelled and locomotry were observed as behavioural traits.
Survival was recorded biweekly.

Test concentrations: 2.5% (12 g/L) glyphosate

Application / device: Residual exposure: spraying equipment not specified; freshly sprayed residuals without drying prior to test.
Topical exposure: micropipette.

Verification of dispersion: visually

Validity criteria: None

5. Environmental conditions:

Test medium: Treated (residual exposure) or dry (topical exposure) filter paper in experiments with *P. milvina*.
Layer of Plaster of Paris in experiments with *H. helluo* and *S. quadriceps*.

Temperature / relative humidity: 24°C

Photoperiod: 13 hours

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KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable.**

Comment:

- Survival of *Pardosa* spiders was observed over a period of 60 days with test animals of unknown age at trial initialization. Under such circumstances survival can barely be attributed to treatment only.
- Animals were tested twice to investigate the effect of handling during the trials which has shown a major impact on most of behavioural alterations. This indicates that animals are already disturbed by the experimental design and the observed behavioural traits are not useful to show clear pesticide effects.
- In the residual-exposure experiments, a 1-cm gap was left between the “two” regions making in fact totally 3 regions, ie. the neutral area of the gap, the distilled water area and herbicide area. However, the neutral area was neglected in results even though spiders can be expected to have spent some time in the gap, too. Authors must have added that time to one of the regions (Total time spent of 1800 s per spider) but did not define how they dealt with that issue.
- In the residual-exposure experiments distilled water and herbicide were sprayed each on semicircle areas on the very same filter paper. Filter paper is supposed to soak up liquids while they diffuse in the paper too. The sprayed distilled water and herbicide probably interfuse in the so-called neutral area of the 1-cm gap but this matter is not given in greater details and remains uncertain.

2. Relevance of study: **Not relevant**

Comment: Behavioural traits without clear cutting-edge results as used in this study are not suitable for laboratory experiments. The use of an artificial environment and controlled conditions always means a major disturbance of animals' behaviour making it difficult to determine alterations due to the treatment. For instance, feeding or mating behaviour or reproductive success are essential traits which can be indeed investigated in the laboratory despite an artificial environment. But locomotion or speed hardly produce robust data under such an experimental design

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

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Author(s)	Year	Study title
Griesinger, L.M., Evans, S.C., Rypstra, A.L.	2011	Effects of a glyphosate-based herbicide on mate location in a wolf spider that inhabits agroecosystems. Chemosphere Volume: 84 Pages: 1461 - 1466 DOI: 10.1016/j.chemosphere.2011.04.044 ISSN: 0045-6535 (print)

Abstract[74]

Chemical communication is important to many arthropod species but the potential exists for anthropogenic chemicals to disrupt information flow. Although glyphosate-based herbicides are not acutely toxic to arthropods, little is known regarding their effects on natural chemical communication pathways. The wolf spider, *Pardosa milvina*, is abundant in agroecosystems where herbicides are regularly applied and uses air- and substrate-borne chemical signals extensively during mating. The aim of this study was to examine effects of a commercial formulation of a glyphosate-based herbicide on the ability of males to find females. In the field, virgin females, when hidden inside pitfall traps with herbicide, attracted fewer males than females with water. Likewise females in traps with a ring of herbicide surrounding the opening were less likely to attract males than those in traps surrounded by water. We explored the reaction of males to any airborne component of the herbicide in a laboratory two-choice olfactometer experiment. When no female pheromones were present, males were equally likely to select herbicide or water treated corridors and they all moved through the apparatus at similar speeds. When female pheromones were present, the males that selected control corridors moved more slowly than those that selected herbicide and, if we control for the initial decision time, more males selected the control corridors over the herbicide. These data suggest that glyphosate-based herbicides are ‘‘info-disruptors’’ that alter the ability of males to detect and/or react fully to female signals.

MATERIALS AND METHODS

1. Test material:

Test item: Buccaneer Plus© registered with US-EPA as Roundup® II Original.

Active substance(s): Glyphosate (*N*-(phosphonomethyl)glycine) isopropylamine salt

Adjuvant: 59% other ingredients including polyethoxylated tallow amine (POEA)

Description: Recommended concentration is 0.625-5% for use in agricultural fields.

Source of test substance: Commercially purchased (manufactured by Monsanto, St. Louis, MO, USA)

Lot/Batch #: Not stated

Purity: 41% (480 g/L)

2. Vehicle and/or positive control: None

3. Test organism:

Species: *Pardosa milvina* (Araneae: Lycosidae)

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[74] Quoted from article

Source: Field-collected from corn and soybean fields at the Miami University Ecology Research Center (Oxford, Ohio, USA)

Age of test organisms at study initiation: 1 week after molting to adulthood

Holding conditions prior to test: Spiders were collected from April through July 2009 and 2010, housed individually in translucent plastic cups containing a thin layer of 50:50 peat moss:soil mixture and fed 2 crickets half the size of the spiders per week. They were held in an environmentally controlled room at 25°C and 13L:11D photoperiod.

Acclimation: Olfactometer experiment: 5 minutes acclimation period in the readily prepared apparatus and airflow switched on.

4. Test system:

Study type: Field (pitfall) and laboratory (olfactometer) experiments

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: Pitfall experiment: 7 h each run (over a period of 6 days)

Olfactometer experiment: max. of 20 min. each observation

Test conditions: Pitfall experiment: 25 pitfall traps were evenly distributed on a no-till soybean field. Traps consisted of a plastic cup surrounded by a ring of filter paper. A mesh-capped vial was inside each cup containing a circle of filter paper. A female was confined in the vial of each cup in treatments but not in the control. Traps were open for 7 hours (10:00 – 17:00) at 5 runs in July 2009. Numbers of trapped males were recorded and categorized as 0, 1, 2, 3 or more males trapped.

Olfactometer experiment: 2-way olfactometer constructed from Plexiglass in which both corridors ended in front of the very same female. A miniature fan behind the female produced the airflow in the olfactometer carrying airborne female cues. The first 7 cm of each corridor was assigned as “choice areas” (i.e. male made a choice by entering this area), followed by “treatment area” of 4 cm (i.e. treated with glyphosate or distilled water) and a final section beyond.

Treatments: Pitfall experiment: 5 different treatments with female and filter paper in vial was treated with distilled water (1) or glyphosate (2), with female and surrounding filter paper ring was treated with distilled water (3) or glyphosate (4) and a control treatment without a female (5)

Olfactometer experiment: 2 different treatments with females present or absent

Replicates per treatment: Pitfall experiment: totally 25 with 5 replicates per treatment at 5 separate experimental runs

Olfactometer experiment: 27

Individuals per replicate: Olfactometer experiment: 1

Parameters measured: Pitfall experiment: Categories of captured *P. milvina* males per pitfall trap (i.e. 1, 2, 3 or more males).

Olfactometer experiment: choice, time needed to reach the areas “choice”, “treatment” and “final”.

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Test concentrations: Pitfall experiment: 2.5% (12 g/L) glyphosate salt
Olfactometer experiment: 1.6% (7.68 g/L) glyphosate salt.

Application / device: Not stated

Verification of dispersion: Not stated

Validity criteria: None

5. Environmental conditions:

Test medium / Soil at study site: Pitfall: Not stated.
Olfactometer: filter paper

Weather conditions / Temperature: Pitfall: Not stated.
Olfactometer: 24°C

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment:

- In pitfall experiments, there is no information about weather conditions or further environmental conditions, like the soil or growth stage of plants on the field.
- Spray history of plants in fields used for pitfall experiments or for the collection of spiders for the experiments was not described in the methods.
- One of the most critical issues in olfactometer experiments is to provide a uniform airflow and avoid turbulence particularly in areas where the animals are supposed to make their choice. Here, a self-constructed design was used with a simple miniature fan in front of both ends without giving information about the exact wind speed and uniformity of airflow inside the two corridors. Validation by observing smoke movement is insufficient. From the diagram in Fig. 1 turbulences can be indeed expected in the entry area where both corridors merge.
- Authors defined the choice area right at the beginning of the two corridors even though this is uncommon in experiments on airborne cues. The animals often change their mind and go the other way in such experiments and especially when there is no uniformity of the airflow. The experimental design and definition of areas does not seem to be reliable.
- No analytical verification of dose
- No dose-response relationship
- No positive control was included to demonstrate the validity of the assay
- Relevant methodological deficiencies (actual doses)
- Method not validated

2. Relevance of study: **Not relevant**

Comment: There is some relevance in pitfall experiments showing a lower attraction in the presence of glyphosate. Reliability of the data

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is decreased due to the lack of information about environmental conditions.

Olfactometer experiments are not state-of-the-art and provide no relevant data.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

Additional information from Ecotox Review

Experimental Conditions and Design -

- Spray volume used to dose the vials (5 µl) in the pitfall experiment was extremely low, and the method used to apply the dose for the pitfall experiment was not described.
- Calculated application rates based on the dose volume, dilution factor, and area dosed were at or well above approved label use rates. The actual calculated rates for the vials and rings in the pitfall experiment were 1.9 and 10.4 kg/ha, respectively, and 11.5 kg/ha for the corridor experiments.
- Use rate doesn't account for crop interception. Crop stage will determine the amount of the applied rate that is intercepted and thus is a determining factor for the exposure of ground dwelling arthropods.
- Spiders were collected from agricultural fields for the experiments from April through July of 2009 and 2010, which is when typical field applications of herbicide would be sprayed. No prior spray history or potential exposure of the spiders used in these experiments is mentioned in the manuscript.
- No positive control substance was concurrently run with either of these experiments.
- Analytical verifications of the actual dose contained on the filter paper were not conducted.

Environmental Relevance -

- Field applications of glyphosate do not occur at the calculated rates used in two of the three trials (10.4 and 11.5 kg/ha).

- $Application\ Rate\ \left(\frac{kg}{ha}\right) = \frac{0.005\ ml \times \frac{12mg}{ml}}{3.1459\ cm^2} \times 100 = \frac{1.91 kg}{ha}$

- $Application\ Rate\ \left(\frac{kg}{ha}\right) = \frac{0.926\ ml \times \frac{12mg}{ml}}{106.5\ cm^2} \times 100 = \frac{10.4\ kg}{ha}$

$$Application\ Rate\ \left(\frac{kg}{ha}\right) = \frac{0.300\ ml \times \frac{7.68mg}{ml}}{20\ cm^2} \times 100 = \frac{11.5\ kg}{ha}$$

- Juvenile spiders were collected from agricultural fields with an apparent prior treatment history with glyphosate, indicating successful reproduction and population recruitment under field application conditions and spider adaptability to cropping systems.
- Only 10 out of 25 pitfall traps in the control group contained a male spider, while 12, 17, 14, and 6 traps in the 2 water and 2 herbicide treatment groups contained at least 1 male spider, respectively. The reliability of this endpoint and sampling design are not well established.
- In-field effects are generally not considered in a risk assessment due to other in-field habitat alterations resulting from agricultural practices.
- The authors state in the conclusions that "*The effects of glyphosate-based herbicides on the behavior of predatory arthropods reported thus far are small and likely to be short-lived. By itself, the info-disruption uncovered here probably has a minor impact on population and communities of spiders in the fields, especially when compared to the changes caused by the dramatic shifts in the plant communities that result from herbicide application.*"

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Author(s)	Year	Study title
Mirande, L., Haramboure, M., Smagghe, G., Pineda, S., Schneider, M.I.,	2010	Side-effects of Glyphosate on the life parameters of *Eriopis connexa* (Coleoptera: Coccinelidae) in Argentina. Communications in Agricultural and Applied Biological Sciences. Volume: 75 Issue: 3 Pages: 367-372 DOI: not stated ISSN: 1379-1176

Abstract[75]

In Argentina, transgenic soybean crop (Roundup Ready, RR) has undergone a major expansion over the last 15 years, with the consequent increase of glyphosate applications, a broad-spectrum and post emergence herbicide. Soybean crops are inhabited by several arthropods. *Eriopis connexa* Germar (Coleoptera: Coccinelidae) is a predator associated to soybean soft-bodies pest and have a Neotropical distribution. Nowadays, it is being considered a potentially biological control agent in South America. The objectives of this work were to evaluate the side-effects of glyphosate on larvae (third instar) and adults of this predator. Commercial compound and the maximum registered concentrations for field use were employed: GlifoGlex 48 (48% glyphosate, 192 mg a.i./litre, Gleba Argentina S.A.). The exposure was by ingestion through the treated prey (*Rophalosiphum padi*) or by drinking treated water during 48 h for treatment of the adult. The herbicide solutions were prepared using distilled water as solvent. The bioassays were carried out in the laboratory under controlled conditions: 23 +/- 0.5 degrees C, 75 +/- 5% RH and 16:8 (L:D) of photoperiod. Development time, weight of pupae, adult emergence, pre-oviposition period, fecundity and fertility were evaluated as endpoints. Larvae from glyphosate treatment molted earlier than controls. In addition, the weight of pupae, longevity, fecundity and fertility were drastically reduced in treated organisms. The reductions were more drastic when the treatments were performed at the third larval stage than as adult. The reproduction capacity of the predator was the most affected parameter and could be related to a hormonal disruption by glyphosate in the treated organisms. This work can confirm the deleterious effects of this herbicide on beneficial organisms. Also, it agrees with prior studies carried out on other predators associated to soybean pest, such as *Chrysoperla externa* (Neuroptera: Chrysopidae) and *Alpaida veniliae* (Araneae: Araneidae).

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KLIMISCH EVALUATION

1. Reliability of study: **Not assignable.**

Comment: This article is an abstract of a conference proceeding (62nd International Symposium on Crop Protection, 18.05.2010, Ghent, Belgium). It summarizes the impact of a formulation containing glyphosate on terrestrial non-target arthropods without giving details about material and methods allowing no evaluation of the reliability of the results.

2. Relevance of study: **Not relevant**

Comment: It is not calculate what level the test organisms were exposed to. Also, the formulation used for toxicity testing seems to differ substantially from the formulation considered as lead formulation for the current submission, although no specifications are provided.

[75] Quoted from article

3. Klimisch code: **Klimisch rating of 4.**

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Effects on the soil microbial community

Microbial populations and associated biochemical processes are critical to maintaining soil health and quality. Soil microbial communities are highly complex and are often characterized by high microbial diversity (Tiedje *et al.* 1999). The occurrence and abundance of soil microorganisms are affected by 1) soil characteristics like tilth, organic matter, nutrient content, and moisture capacity, 2) typical physico-chemical factors such as temperature, pH, and redox potential, and 3) soil management practices. Agricultural practices such as fertilization and cultivation may also have profound effects on soil microbial populations, species composition, colonization, and associated biochemical processes (Buckley and Schmidt 2001; Buckley and Schmidt 2003). Consequently, significant variation in microbial populations is expected in agricultural fields. Minor changes in a single microbial species or group are difficult to measure in such a dynamic system and, moreover, the minor effects of such a change may be better assessed in more integrated measures such as soil fertility and other soil processes such as nitrogen metabolism and community respiration. Research on the effects of agricultural practices on soil microbial communities should consider interactive effects of soil ecology to aid in interpretation of study results.

Effects of Roundup Agricultural Herbicides on Soil Microbes

The effects of glyphosate and glyphosate-based formulations on soil microorganisms have been extensively investigated (Cerdeira and Duke, 2010; Sullivan and Sullivan, 2000). Results of standardized tests with glyphosate formulations performed for submission to regulatory agencies indicate no long-term effects on microorganisms in soil even at rates that exceed maximum use rates (up to five times the labeled rate). In addition, independent researchers have reviewed numerous laboratory and field studies, investigating the effects of glyphosate on soil bacteria and fungi (Felsot, 2001; Giesy et al., 2000). In a recent greenhouse investigation, Arango (2009) evaluated the effects of multiple glyphosate applications on the rhizosphere bacterial community associated with glyphosate-tolerant soybean. While subtle, transient shifts in community structure were noted after glyphosate applications, effective resilience and no reduction in bacterial diversity were observed for the bacterial community associated with roots of glyphosate-treated versus unsprayed glyphosate-tolerant soybean. Although some laboratory tests have shown effects on nitrogen-fixing bacteria (Moorman et al., 1992; Santos and Flores, 1995) and soil fungi (Estok et al., 1989; Busse et al., 2001), effects are typically observed only under artificial laboratory conditions and at glyphosate concentrations well above normal field application rates. Several researchers have concluded that it is difficult to extrapolate results from the laboratory to the natural soil environment (Estok et al., 1989; Wan et al., 1998; Busse et al., 2001).
Investigations by Haney et al. (2000, 2002) related to the increased use of glyphosate-tolerant crops indicate that glyphosate was degraded over time by soil microbes, even at high application rates, without adversely impacting the soil microbial community. In addition, results from field studies that have evaluated the fungal component of the soil microbial community indicate that glyphosate treatment had no deleterious effects on beneficial soil fungi (Araujo et al., 2003; Biederbeck et al., 1997; Busse et al., 2001; Wardle and Parkinson, 1990a, b). In a 4-year field study, Powell et al. (2009) assessed effects of glyphosate applications on soil food web properties and crop litter decomposition in a glyphosate-tolerant soybean and maize rotation. The researchers concluded that: “Permanent responses in soil biota were not observed, suggesting a high level of resilience in the soil biota and a lack of a persistent effect resulting from the GM cropping system.” Furthermore, Liphadzi et al. (2005) observed that the soil microbial and nematode community was similar when glyphosate or conventional herbicides were applied to crop rotations of glyphosate-tolerant soybean and maize cultivars. Moreover, the history of safe use and yield data obtained for nearly 15 years of glyphosate-tolerant crop production, combined with in-crop applications of glyphosate-based agricultural herbicides, reinforce the findings that soil microbes and microbially mediated processes are not adversely impacted by field-rate applications of glyphosate.

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Glyphosate Effects on Plant Disease and Nutrient Status

Several research groups have reported that use of glyphosate, particularly on Roundup Ready crops, has deleterious effects on plant health, especially plant nutrient status and disease resistance. A comprehensive analysis of this subject matter is covered in the environmental fate chapter under section IIA 7.13.
The important points in this section are:

- The mode of action of glyphosate is well understood and does not depend on Mn chelation or disease susceptibility.
- The success and fitness of Roundup Ready crops indicates that the effects of Mn chelation by glyphosate on plant physiology are minor.
- The current recommendations for tank mixes of glyphosate and Mn fertilizers take into account the possibility of Mn chelation by glyphosate in the solution.
- The levels of disease observed in glyphosate-treated Roundup Ready crops are variable: both increases and decreases have been reported. Effects on disease can be either direct or indirect.
- The effect of glyphosate on soil microbes is similarly variable, with both increases and decreases in populations being reported in short-term studies. Long-term field studies have not shown a significant difference in populations.
- The available data do not support a reduction in glyphosate use rates as a means of reducing plant disease.
- The Roundup Ready soybean production system is not expected to negatively affect soil fertility, nodulation, or nitrogen fixation.

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Effects on earthworms and other soil macro-organisms

Overview

Earthworms are important components of agricultural ecosystems, and the impact of agricultural practices has been extensively reviewed by other scientific, ecological and agricultural organizations. The definitive work on earthworms reviewed the effects of many agricultural products on earthworms (Edwards and Bohlen, 1996). Glyphosate was ranked as zero on a scale of zero (relatively non-toxic) to 4 (extremely toxic).

Laboratory Studies

The Glyphosate Task Force has conducted several 14-day studies which demonstrate that glyphosate, the lead formulation and additional glyphosate formulations are harmless to earthworms (*Eisenia foetida*) at concentrations greatly exceeding what would be expected through normal application of the product (see section IIIA 10.6). These studies were conducted according to standard protocol and established Good Laboratory Practices. In addition to short-term studies, the effects of glyphosate and its soil metabolite AMPA (aminomethyl phosphonic acid) on reproduction in the earthworm over a 56-day exposure period have been investigated and are reported in section IIA 8.9. No effects on growth or reproduction of the earthworm *Eisenia foetida* were observed at greater than fie times soil concentrations observed under actual use conditions. Additionally, new studies, following international guidelines, demonstrate very low risk to glyphosate and AMPA to other soil macro-organisms at concentrations greater than five times worst-case field exposure conditions.

Conclusions

A wealth of information from years of experience and credible studies supports the conclusion that normal use of glyphosate formulations, such as Roundup, will not result in adverse effects to earthworms.

Literature cited

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Author(s)	Year	Study title
Correia F. V, Moreira J. C.	2010	Effects of Glyphosate and 2,4-D on Earthworms (*Eisenia foetida*) in Laboratory Tests Bulletin of Environmental Contamination and Toxicology Volume: 128 Issue: - Pages: electronic publication DOI: 10.1007/s00128-010-0089-7 ISSN: 0007-4861 (print),1432-0800 (web)

Abstract[76]

Laboratory tests were conducted to compare the effects of various concentrations of glyphosate and 2,4-D on earthworms (*Eisenia foetida*) cultured in Argissol during 56 days of incubation. The effects on earthworm growth, survival, and reproduction rates were verified for different exposure times. Earthworms kept in glyphosate treated soil were classified as alive in all evaluations, but showed gradual and significant reduction in mean weight (50%) at all test concentrations. For 2,4-D, 100% mortality was observed in soil treated with 500 and 1,000 mg/kg. At 14 days, 30%–40% mortality levels were observed in all other concentrations. No cocoons or juveniles were found in soil treated with either herbicide. Glyphosate and 2,4-D demonstrated severe effects on the development and reproduction of *Eisenia foetida* in laboratory tests in the range of test concentrations.

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MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate [N-(phosphonomethyl)glycine]
Active substance(s): Glyphosate
Adjuvant: none
Description: none
Source of test substance: Sigma Chemical
Lot/Batch #: Not stated
Purity: 99.7%
Stock solution: Not stated

2. Vehicle and/or positive control: none

3. Test organism:

Species: *Eisenia fetida,* formerly known as *E. foetida*
Source: Minhocário Arborium (RJ)
Age of test organisms at study initiation: Less than 2 month, clitella well developed
Weight: 300 – 600 mg
Holding conditions prior to test: 4 weeks at 20°C ± 2 in bovine manure

[76] Quoted from article

Acclimatisation: none

4. Test system:

Study type: Single species test in laboratory
Guideline: none
GLP: Not stated
Guideline deviations: none
Duration of study: 56 days
Test conditions: Natural soil
Water volume/ Soil moisture: 60 % of maximum water holding capacity
Treatments: 5
Replicates per concentration: 4
Individuals per replicate: 10
Parameters measured: Survival, mortality, biomass (loss of weight in %), Abundance of cocoons and juveniles
Test concentrations: 1, 10, 100, 500, 1000 mg/kg
Application / device: Not stated
Verification of dispersion: Not stated
Validity criteria: Not stated

5. Environmental conditions:

Test medium / Soil at study site: classified as Argissol[77]: 608 g/kg sand; 112 g/kg silt; 280 g/kg clay; 10.8 g/kg organic carbon;
Weather conditions / Temperature / relative humidity: 20 ± 2 °C
Feeding: Not reported
Photoperiod: Continuous light, intensity not stated
pH: pH 5.5 (prior to spiking)
Organic matter (C_{org}): 10,8 g/kg
$CaCO_3$ Not stated
Cation exchange capacity: Not stated
Soil textural fractions / extractable micronutrient concentrations [mg per kg soil]: Not stated
Fertilization: Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:
- The study was conducted using natural soil with a low proportion of organic matter. *E. fetida* as an epigeic earthworm species prefers a substrate with high organic content (manure, compost). Although *E. fetida* may survive under these hostile conditions, it will

[77] EMBRAPA – Empresa Brasileira de Pesquisa Agropecua´ria (1997) Centro Nacional de Pesquisa em Solos. Manual de me´todos de ana´lise de solo, 2a. ed. Rio de Janeiro, 212p

definitely suffer starvation and lose weight, let alone with no additional feeding.

- Extremely low fecundity reported for the controls (12 cocoons and 6 juveniles for 80 worms), in comparison to the requirements of e.g. OECD 222, which requires each replicate (containing 10 adults) to have produced ≥ 30 juveniles by the end of test (also 56 days).
- Results for lowest exposure concentration not presented.
- Before exposure earthworms were kept for four weeks in cattle manure, a substrate consisting almost exclusively of organic matter, in contrast to the exposure substrate.
- Observations such as shedding of cuticles indicate that the moisture content was not maintained throughout the exposure period (worms dried out).
- pH value is only reported for the native soil, but not for the spiked samples. Injuries of adult earthworms reported especially for 2,4-D, but also, to a minor extent for glyphosate, suggest that the pH was not stable during exposure.

2. Relevance of study:

Comment: Test setup roughly comparable to OECD 222/ISO 11268-1/2. However, due to substantial flaws within the experimental setup, the findings are considered not reliable and in are in contrast to the weight of evidence of the standard regulatory GLP studies.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment**

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Effects on nontarget plants

A number of studies that assessed the effect of glyphosate on seed germination and vegetative vigor have been published the 2001 EU glyphosate evaluation. The majority of these studies tested rates that exceed well accepted drift rates and effects. In sections IIA 8.12, IIIA 10.8.1.1 and IIIA 10.8.1.2 the non-target plant data is summarized and in section IIIA 10.8.1.2 is a completed ecological risk assessment for non-target plant communities.

Author(s)	Year	Study title
Blackburn, L:G., Boutin, C.	2006	Subtle effects of Herbicide Use in the Context of Genetically Modified Crops. Ecotoxicology. Volume: 12 Pages: 271-285 DOI: ISSN: 0963-9292 (print), 1573-3017 (online)

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Abstract[78]

Research on the effects of glyphosate and seed germination spans 30 years. Despite several studies reporting detrimental effects of the herbicide on seedling germination and growth, glyphosate is still being registered for use as a weed killer and preharvest desiccant. Its nonselective nature and low chance of species developing resistance has lead to the development of genetically modified crops tolerant to the herbicide which also raises concerns about increased reliance on herbicide use, and subtle ecological impact.
This paper presents the result of a literature review on past studies mostly, on crop species, and the results of a new experiment performed with emphasis non noncorp species. The new experimental part attempted to determine whether glyphosate (Roundup®) would have an effect on the germination and growth of the F1 generation of seeds produced by plants sprayed with the herbicide. It was designed to mirror spray drift which might affect noncrop plants in nontarget drift zones. Of the 11 species tested using treatments of 0% (control), 1%, 10% or 100% of 890 g a.i./ha label rate solution sprayed near seed maturity, seven showed a significant effect of the glyphosate treatment on germination and/or growth characteristics.
Results of this experiment together with several previous studies reviewed in this paper suggest that there are significant effects to keep in mind when using herbicides such as glyphosate as severe ecological changes could occur.

MATERIALS AND METHODS

References:

Publication period of reviewed paper: 1970 - 2002

KLIMISCH EVALUATION

1. Reliability of study: **Not assignable.**

Comment: This article is a literature review allowing no evaluation of the reliability.

2. Relevance of study:

[78] Quoted from article

Comment: The article evaluated herewith reviewed eighteen previous studies with glyphosate application to thirteen different plant species including wheat and conducted original research on eleven species including wheat, ten of which were species not reported in the previous studies in Table 1 of the paper. In one of the eighteen previous studies, rates at which effects occurred were not reported, and this study could not be evaluated further. For sixteen of the seventeen remaining studies, all of the effects of glyphosate listed in Table 1 occur at application rates greater than the 5% of the application rate (0.087 kg a.e./ha); and for the eighteenth study, all effects occurred at rates above 1% of the maximum application rate of 1.74 kg a.e./ha).

For the original research reported in the paper, plants were either untreated or treated with glyphosate at 1%, 10% or 100% of the application rate of 0.89 kg a.e./ha during the seed development stage. After some further development, these seeds were harvested and germinated either between moist filter paper in a Petri dish or in soil. Seedling growth was then measured. The overall conclusions from this work were: “While results of statistical analyses indicate an effect of glyphosate concentration on some species, most effects were due to exposure to the 100% label rate ....” The 100% label rate described in this quotation is 0.89 kg a.e./ha, which is at least an order of magnitude above the exposure anticipated to result from drift at typical glyphosate use rates. A review of the results reported in the paper indicates that the effects that were noted from glyphosate applications at rates below 100% were generally inconsistent between the two methods that were used for testing (germination and seedling growth in a Petri dish vs germination and seedling growth in soil). This inconsistency is observed for wheat, and effects from the more realistic method (germination in soil) did not indicate that there were effects on growth. In summary, for the 23 species for which results have been summarized in this paper, exposure to glyphosate at rates anticipated due to drift from ground applications are not expected to impact these species.

3. Klimisch code: **Klimisch rating of 4.**

Author(s)	Year	Study title
Boutin, C., White, A. L., Carpenter, D.	2010	Measuring variability in phytotoxicity testing using crop and wild plant species Environmental Toxicology and Chemistry Volume: 29 Issue: 2 Pages: 327–337 Url: http://www.ncbi.nlm.nih.gov/pubmed/20821451 DOI: 10.1002/etc.30 ISSN: 0730-7268 (print), 1552-8618 (online)

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Abstract[79]

A series of experiments was conducted to assess the level of variability in phytotoxicity testing and to investigate factors that may explain some of the observed uncertainties and inconsistencies. The work was conducted in greenhouse or growth chamber environments with plants growing individually in pots and harvested 28 d after spraying with two herbicides, glyphosate and atrazine, as formulated products. Between six and 10 doses were used on five or six replicates, necessitating over 4,500 individually growing plants. In the first set of experiments, several ecotypes (originating from different areas of the world) of eight wild plant species were tested. Significant differences in sensitivity to atrazine and glyphosate were found among ecotypes of most species tested. In the second suite of experiments, the reproducibility of results during different seasons (when growing conditions vary) was investigated using three crops and four wild plant species. Results showed that seasonal variability elicited a pronounced discrepancy in response between plants tested at different times of the year. It was found that no consistent effects could be attributed to the biotic or abiotic factors investigated. Several ecotypes of the same species differed in their seed size, percentage germination, or germination requirements, as well as in growth patterns, but these differences could not explain differences in herbicide sensitivity. Likewise, differences in phytotoxicity could not be attributed to factors such as temperature, light intensity, and sunlight duration. The present study supports the inclusion of an uncertainty factor in risk assessments to account for the intrinsic variability in plant sensitivity to herbicides.

MATERIALS AND METHODS

1. Test material:

Test item:	Round-Up Original® or Vision®
Active substance(s):	Glyphosate
Surfactant:	Nonylphenoxy polyethyoxyethanol (Agral 90, Norac Concepts)
Description:	
Source of test substance:	Monsanto Canada
Lot/Batch #:	Not stated
Purity:	356g/L glyphosate [N-(phosphonomethyl) glycine]

2. Vehicle and/or positive control:

3. Test organism:

Species: <u>Ecotype sensitivity experiment</u>

Bellis perennis L.

[79] Quoted from article

Centaurea cyanus L.
Digitalis purpurea L.
Inula helenium L
Prunella vulgaris L.
Rumex crispus L.
Rudbeckia hirta L.
Solidago canadensis L.

Temporal variability experiment
Lycopus americanus Muhl.
Geum canadense Jacq.
Chrysanthemum leucanthemum L.
Triticum aestivum L.
Lactuca sativa L.
Solanum lycopersicon L.

Age of test organisms at study initiation:	Three- to five-leaf stage
Source:	Europe and North America, from commercial seed suppliers, donated or wild populations
Holding conditions prior to test:	test with pesticides: not specified germination test 0- or 1-month stratification period coupled with two different environments (a greenhouse and a growth chamber, which exhibited differences in light intensity and temperature fluctuations)

4. Test system:

Study type:	Greenhouse or growing chambers
Guideline:	none
GLP:	Not stated
Guideline deviations:	-
Duration of study:	28 d
Test conditions:	Plants were grown from seed and transplanted into 10-cm diameter by 9-cm-height plastic pots containing a 3:1 Promix BX with Mycorise1 Pro (Premier Horticulture) soil:sand mixture within 14 d of germination
Replicates per concentration:	six (ecotype variability experiment) six (temporal variability experiment)
Organisms per replicate:	1 plant per pot. Typically nontarget plant studies have multiple plants per replicate to reduce variability.
Parameters measured:	Biomass (dry weight), germination (test without herbicide), seed size and number.
Test concentrations:	Ecotype variability experiment Nine or ten doses both following a geometric progression of 1.7 from 21 to 2,277 g a.i./ha

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Temporal variability experiment:
Six to nine doses following a geometric progression ranging from 1.3 to 1.6 (53 to 2,285 g a.i./h)

Application: 6.75 ml/m^2 at 206.84 kPa

Application devices: a track spray booth (de Vries Manufacturing) equipped with a TeeJet 8002E flat-fan nozzle (Spraying Systems)

Application verification: not stated

Analytical determination of test concentrations: not stated

5. Environmental conditions:

Test medium: 3:1 Promix BX with Mycorise Pro (Premier Horticulture) soil:sand mixture

Temperature: 16 – 43°C

Photoperiod: 16 h daylight

Light intensity: 106 – 1959 µmol m^{-2} s^{-1} (photosynthetically active radiation (PAR))

pH: not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:
- Source of seeds are not precisely reported, only information about the country of origin are available
- Data on physical and chemical test conditions are missing
- No information about the verification of the application (e.g., measured concentration of glyphosate in spray solutions)
- No information about the watering regime and nutrient content of the soil
- ecotypes for the temporal variability experiment are not documented
- well documented: statistical methods/ results, effect concentrations

2. Relevance of study: **Relevant with restrictions**

Comment: Some useful information are missing (see above), but dose response curves are presented including effect concentrations (IC_{25} = 25% inhibition concentration), but no IC_{50} value which is the critical endpoint used in the EU non target plant risk assessments. IC_{25} values for biomass reduction on 14 species of plant for glyphosate ranged from 60 g a.s./ha to 1400 g a.s./ha in different tests and on different species. These endpoints are comparable to the range of IC_{25} values obtained in glyphosate data as submitted and summarized in section 8.12. The EC_{50} values would be higher.

As a non selective herbicide care should be taken to avoid spray drift when using this product. The lowest measured IC_{25} value for glyphosate in this study represents 2.8% of the

maximum application rate for use in broadcast applications of 2160 kg a.s./ha. The standard model for drift exposure in the EU would predict the drift exposure level to be 2.77% of the application rate at 1m from the edge of the field, indicating that accidental spray drift from glyphosate application will represent a low risk to most species of plants and non target plant populations situated more that 1m from the sprayed target areas.

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
Ding, W., Reddy, K.N., Zablotowicz, R.M., Bellaloui, N., Bruns, H.A.	2011	Physiological responses of glyphosate-resistant and glyphosate-sensitive soybean to aminomethylphosphonic acid, a metabolite of glyphosate. Chemosphere Volume: 83 Issue: 4 Pages: 593-598 Url: http://www.sciencedirect.com/science/article/pii/S0045653510013986 DOI: 10.1016/j.chemosphere.2010.12.008 ISSN: 0045-6535

Abstract[80]

Aminomethylphosphonic acid (AMPA) is formed in glyphosate-treated glyphosate-resistant (GR) and glyphosate-sensitive (GS) soybean [*Glycine max* (L.) Merr.] plants and is known to cause yellowing in soybean. Although, AMPA is less phytotoxic than glyphosate, its mode of action is different from that of glyphosate and is still unknown. Greenhouse studies were conducted at Stoneville, MS to determine the effects of AMPA on plant growth, chlorophyll content, photosynthesis, nodulation, nitrogenase activity, nitrate reductase activity, and shoot nitrogen content in GR and GS soybeans. AMPA was applied to one- to two-trifoliolate leaf stage soybeans at 0.1 and 1.0 kg ha^{-1}, representing a scenario of 10% and 100% degradation of glyphosate (1.0 kg ae ha^{-1} use rate) to AMPA, respectively. Overall, AMPA effects were more pronounced at 1.0 kg ha^{-1} than at 0.1 kg ha^{-1} rate. Visual plant injury (18–27%) was observed on young leaves within 3 d after treatment (DAT) with AMPA at the higher rate regardless of soybean type. AMPA injury peaked to 46–49% at 14 DAT and decreased to 17–18% by 28 DAT, in both soybean types. AMPA reduced the chlorophyll content by 37%, 48%, 66%, and 23% in GR soybean, and 17%, 48%, 57%, and 22% in GS soybean at 3, 7, 14, and 28 DAT, respectively. AMPA reduced the photosynthesis rate by 65%, 85%, and 77% in GR soybean and 59%, 88%, and 69% in GS soybean at 3, 7, and 14 DAT, respectively, compared to non-treated plants. Similarly, AMPA reduced stomatal conductance to water vapor and transpiration rates at 3, 7, and 14 DAT compared to non-treated plants in both soybean types. Photosynthesis rate, stomatal conductance, and transpiration rate recovered to the levels of non-treated plants by 28 DAT. Plant height and shoot dry weight at 28 DAT; nodulation, nitrogenase activity at 10 DAT, and nitrate reductase activity at 3 and 14 DAT were unaffected by AMPA. AMPA reduced root respiration and shoot nitrogen content at 10 DAT. These results suggest that a foliar application of AMPA could indirectly reduce photosynthesis through decreased chlorophyll content in GR and GS soybean up to 14 DAT, but affected plants can recover to normal growth by 28 DAT.

MATERIALS AND METHODS

1. Test material:

Test item:	Amino-methylphosphonic acid (AMPA)
Active substance(s):	AMPA
Adjuvant:	Tween 20 (0.5%, v/v)
Description:	none
Source of test substance:	AMPA: Sigma-Aldrich, Allentown, PA, USA Tween: Sigma-Aldrich, St. Louis, MO, USA
Lot/Batch #:	Not stated

[80] Quoted from article

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Purity: >99% (techn. grade)

2. Vehicle and/or positive control:

3. Test organism:

Species: *Glycine max* - Glyphosate-resistant (GR) cultivar AG4605RR/S and non-GR soybean cultivar Williams 82

Source: Soybean cultivars' source not stated and bacteria *B. japonicum* was an abundant native population at study site.

Age of test organisms at study initiation / Crop growth stage at treatment: About 20 days after sowing at 1- or 2-trifoliolate leaf growth stage.

Holding conditions prior to test: Seeds were planted in 15-cm diameter pots containing 1:1 (v/v) mixture of Bosket sandy loam soil and Dundee silty clay loam soil and sub irrigated with distilled water as needed. After emergence plants were thinned to 2 plants per pot and kept in greenhouse at 28/22 °C (±3 °C) day/night with 13-h photoperiod with natural light supplemented by sodium-vapour lamps.

4. Test system:

Study type: Greenhouse

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 28 days after application (plant injury, height, shoot dry weight, chlorophyll and photosynthesis measurements)
10 days after application (nodulation, acetylene reduction assay, respiration, shoot and root biomass and shoot nitrogen)
14 days after application (leaf in vitro nitrate reductase activity)

Test conditions: Study was conducted at USDA-ARS Jamie Whitten Delta States Research Station, Stoneville, MS. Plants were grown in 15 cm diameter plastic pots containing 1.7 kg of 1:1 (v/v) mixture of Bosket sandy loam soil and Dundee silty clay loam soil.

Water volume: Appr. 190 L/ha

Treatments: AMPA spray solutions were prepared using AMPA and Tween 20 at 0.5% v/v, control plants were sprayed with water and 0.5% v/v Tween. Spray solutions were applied using an indoor spray chamber with air-pressurized system at a volume of 190 L/ha at 140 kPa using 8002E flat-fan nozzles.

Replicates per treatment: 6 in all assays except for photosynthesis that had nine replicates.

Individuals per replicate: 2 (plants/pot)

Pot size: 15 cm diameter

Parameters measured: Plant injury, chlorophyll and photosynthesis were determined at 3, 7, 14 and 28 days after treatment. Plant height and shoot dry weight were determined 28 days after treatment.
Nodulation, acetylene reduction assay, respiration, shoot and root biomass and shoot nitrogen were determined at 10 days after treatment.
Leaf in vitro nitrate reductase activity was determined 3 and 14 days after treatment.

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	Data were subjected to ANOVA followed by Fisher's LSD test.
Test concentrations:	0.1 and 1.0 kg AMPA/ha, representing a scenario of 10% and 100% glyphosate (single application 1.0 kg/ha) degradation to AMPA.
Verification of dispersion / volume:	Calibration beforehand not reported.
Validity criteria:	None

5. Environmental conditions:

Test medium:	Bosket sandy loam soil: fine loamy, mixed, thermic Mollic Hapludalfs Dundee silty clay loam soil: fine silty, mixed, thermic Aeric Ochraqualf from field under continuous soybean production for 4 years containing an abundant population of *Bradyrhizobium japonicum*.
Temperature:	28/22 °C (±3 °C) day/night
Photoperiod:	13-h photoperiod
Lighting	Natural light supplemented by sodium-vapour lamps
pH:	Not stated
Organic matter (C_{org}):	Not stated
$CaCO_3$	Not stated
Cation exchange capacity:	Not stated
Soil textural fractions:	Not stated
Fertilization:	Not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:
- Verification or calibration of applied volume not stated.
- Environmental conditions in some aspects only vaguely described (e.g. no detailed characterization of physic-chemical conditions in test medium provided in article).
- No validity criteria stated but 2 control treatments for adequate comparisons.

2. Relevance of study: **Not relevant**

Comment: AMPA was co-applied with an adjuvant (Tween 20) which is unrealistic. This study is not relevant for the risk assessment as AMPA is formed in soil and *in planta* production is addressed in standard non-target plant studies.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

Author(s)	Year	Study title
Boutin, C., Elmegaard, N., Kjaer, C.	2004	Toxicity testing of fifteen non-crop plant species with six herbicides in a greenhouse experiment: Implications for risk assessment Ecotoxicology Volume: 13 Issue: 4 Pages: 349-369 Url: http://www.springerlink.com/content/h11p2r43822518u6/ DOI: 10.1023/B:ECTX.0000033092.82507.f3 ISSN: 0963-9292 (Print), 1573-3017 (Online)

Abstract[81]

Estimation of risk to plants not targeted by herbicides when used in agricultural or forestry situations requires appropriate data on multiple species. Currently, many questions remain unresolved as to the adequate type and number of species to be tested. This paper presents the result of a unique greenhouse experiment where testing was performed with 15 non-crop plant species sprayed with 6 herbicides. The herbicides were chosen because of their different modes of action and because they are widely used in several countries. The plants favoured were species commonly found in field margins of Europe and/or North America. This dataset (called thereafter Danish/Canadian) was compared to the crop species that had been submitted to the US EPA for the same herbicides. In general, the selected plant species in the Danish/Canadian database were easy to grow and maintain in the greenhouse. The Danish/Canadian plants were overall more sensitive than the species tested in the US EPA data, yielding to a 5% protection threshold ($HC5_{(50)}$) that was always more conservative. There was a large variability in plant responses among herbicides. Recommendations are provided on species that should and should not be used for risk assessment of non-target plants.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Bio
Active substance(s):	Glyphosate
Surfactant:	Not stated
Description:	Not stated
Source of test substance:	Monsanto
Lot/Batch #:	Not stated
Purity:	360g/L glyphosate with 480 g glyphosate IPA salt
Stock solution:	Immediately before spraying, a stock solution consisting of the highest dosage tested was made up, lower doses were obtained by diluting the stock solution. Stock solution was verified by HPLC.

2. Vehicle and/or positive control: none

3. Test organism:

[81] Quoted from article

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Species: *Bellis perennis* L.
Centaurea cyanus L.
Inula helenium L.
Rudbeckia hirta L.
Solidago canadensis L.
Leonorus cardiaca L.
Mentha spicata L.
Nepeta cataria L.
Prunella vulgaris L.
Polygonum convolvulus L.
Rumex crispus L.
Anagallis arvensis L.
Digitalis purpurea L.
Sinapis arvensis L.
Papaver rhoeas L.

Age of test organisms at study initiation: Range finder: four- to eight-leaf stage

Source: *P. convolvulus* and *S. arvensis* were collected in Denmark, all other species ordered from seed suppliers in the U.S. or Canada.

Holding conditions prior to test: For germination, all species were sown at soil surface in large trays and later transplanted into individual 11 cm (diameter? depth?) pots at early seedling stage.

4. Test system:

Study type: Greenhouse

Guideline: None, similar to OECD 227

GLP: Not stated

Guideline deviations: Fewer plants and fewer replicates used, potting soil instead of sandy loam used.

Range finder: Between three to five plants per pot and two replicates per treatment rate were exposed to four treatment rates plus control (sprayed at 0.01, 0.1, 1 and 5 or 10 times the recommended label rates for agricultural use in Canada and Denmark (1440 g a.e./ha for glyphosate)). Visual observations of effects were made 2 weeks after treatment.

Duration of study: 21 d

Test conditions: Study was performed in a greenhouse at National Environmental Research Institute at Silkeborg, Denmark, between August and December 1998. Plants were watered from the bottom.

Replicates per concentration: 6

Organisms per replicate: 1 plant per pot. Typically nontarget plant studies have multiple plants per replicate to reduce variability.

Parameters measured: Range finder: Visual effects 2 weeks after application.

Definite test: shoot dry weights 3 weeks after application. EC_{50} values were calculated using the linear interpolation method for sublethal toxicity, also called the inhibition concentration approach (ICp), hazardous dosage was determined using the E_TX program 1993.

Test concentrations: Four doses following a geometric progression ranging from 1.8

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to 2.0 and control

Application: 200 L water/ha at 2 bars at 50 cm distance from nozzle

Application devices: Moving boom sprayer with standard flat fan nozzles (Hardi 4110-16 nozzle)

Application verification: not stated

Analytical determination of test concentrations: not stated

5. Environmental conditions:

Test medium: Commercial potting soil with high peat content.

Temperature: 15-25°C

Photoperiod: 16 h daylight

Light intensity: Not stated

pH: not stated

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Source of seeds are not precisely reported, only information about the country of origin are available
- Data on physical and chemical test conditions are missing (e.g. light intensity)
- Control plants were segregated in an area away from the treated plants and may have experienced different environmental conditions.
- No information about the verification of the application (e.g., measured concentration of glyphosate in spray solutions)
- No information about the nutrient content of the soil
- Inconsistent results between the range finding and definitive portions of the study.
- Because the plants were treated at a young growth stage it is uncertain whether the plant root system had time to become established after transplantation before the glyphosate treatment occurred.
- This study deviates from the the OECD Test Guideline 227, Terrestrial Plant Test: Vegetative Vigour Test in several ways. Fewer plants per replicate and fewer treatment rates were used in the Boutin et al. study than prescribed by the regulatory test guidelines. Potting soil rather than sandy loam soil was used. Light intensity during the test was not specified. Control plants were segregated in an area away from the treated plants and may have experienced different environmental conditions. This segregation is unnecessary for glyphosate since it has very low volatility.

2. Relevance of study: **Not relevant**

Comment: Some useful information are missing (see above). For a

substantial number of plant species, the EC_{50} found in the definitive test is below (6 species) the lowest concentration tested or between the lowest and 2nd lowest concentration (11 of 15 species).
Because of the internal inconsistencies in the Boutin et al. paper and the deviation from established test methods, the results from the regulatory studies are most relevant should be utilized for risk assessment, rather than the results from the Boutin et al. study.

3. Klimisch code: **Klimisch rating of 3 and not acceotable for risk assessment.**

Important additional considerations:
Fifteen plant species--five species from the Asteraceae family (daisy family), four from the Lamiaceae family (mint family), two from the Polygonaceae family (buckwheat family), and the rest from four other families were sowed at the soil surface and later transplanted at the early seedling stage. Commercial potting soil with a high peat content was used for the soil. A range-finding test was performed using between three to five plants per pot and two replicates per treatment rate. Four treatment rates plus control were sprayed at 0.01, 0.1, 1 and 5 or 10 times the recommended label rates for agricultural use in Canada and Denmark (1440 g a.e./ha for glyphosate). Visual observations of effects were made 2 weeks after treatment. The definitive test, aimed at obtaining rate-response curves, was performed using four rates with a geometric progression of 1.8 to 2.0 as determined by the range-finding test, plus control. Plants were sprayed with a moving boom sprayer with standard flat fan nozzles. For the definitive test 6 replicates with a single plant per pot were used for each treatment rate. Three weeks after treatment, the above ground portion of the plants was removed and weight measurements were taken after drying. EC_{50} values were calculated using the linear interpolation method for sublethal toxicity, also called the inhibition concentration approach (ICp) (as described in US EPA report EPA/600/4/-89-001 and 001A). The range of EC_{50} values from the Boutin et al. study was 14.26 g a.e./ha – 64.66 g a.e./ha.

The Boutin et al. (2004) foliar application study has endpoints that are considerably lower (by approximately 15 times) than three other regulatory nontarget plant studies for glyphosate discussed in the paper. Boutin et al. attribute the difference in sensitivity between their data and that from the regulatory studies to use of a formulated glyphosate product in their study; however, in the U.S. EPA California red-legged frog pesticide effects determination (US EPA, 2008) two of these three regulatory studies are indicated as formulation studies, and the third study had surfactant added to the spray solution. An alternative explanation for the difference in sensitivity between the studies may be significant differences in methodology between the Boutin et al. study and the other studies.

In the Boutin et al. study, seeds of the plant species to be tested were sowed at the soil surface in potting mix with a high peat content, and then transplanted at the early seedling stage prior to herbicide treatment. The seeds were planted on the soil surface because they required sunlight for germination. Because the details of the transplantation are not provided, the potential for root injury can not be assessed. Because the plants were treated at a young growth stage it is uncertain whether the plant root system had time to become established after transplantation before the glyphosate treatment occurred. The extent of growth of the control plants is not reported. The glyphosate binding properties of the potting mix versus sandy loam soil are also unknown. In agricultural soils, glyphosate is bound tightly and does not display herbicidal activity. Whether glyphosate displays herbicidal activity in the high peat potting soil was not determined. Because the methodology used in the Boutin et al. study is not consistent with environmental exposure and was not a standard or common test protocol, the endpoints from Boutin study should not considered for risk assessment.

This study deviates from the EPA OPPTS 850.4250 Vegetative Vigor, Tier II guideline and the OECD Test Guideline 227, Terrestrial Plant Test: Vegetative Vigour Test in several ways. Fewer plants per replicate and fewer treatment rates were used in the Boutin et al. study than prescribed by the regulatory

test guidelines. Potting soil rather than sandy loam soil was used. Light intensity during the test was not specified. Control plants were segregated in an area away from the treated plants and may have experienced different environmental conditions. This segregation is unnecessary for glyphosate since it has very low volatility.

The OECD guideline recommends that rates tested for determination of an EC_{50}, should produce a 20 to 80% effect. The reported EC_{50} values in Boutin et al. for most species are either below the lowest rate tested or between the two lowest rates tested. Because a good rate response is not achieved, the calculation of the EC_{50} is less certain due to extrapolation beyond the limits of the data. Such extrapolation can often lead to erroneous conclusions unless the actual data and goodness-of-fit criteria are included in the report. Because the growth inhibition data are not reported, the shape and fit of the rate response curve can not be determined.

The methodology of this study is also called into question because it gave inconsistent results between the range finding and definitive portions of the study. In seven of the 15 species tested, the calculated EC_{50} was below the lowest rate that gave an effect (LOEL) in the range finding study. Based on information derived from Appendix 1 of the Boutin et al. paper, no visual effects were reported in the range-finding test for *Mentha spicata*, and *Sinapis arvensis* at 14.4 g a.i./ha (LOEL 144 g a.i./ha), but in the definitive test, the EC_{50} values (17.94 and 19.28 g ai/ha, respectively) for these species were very similar to the no effect rate. For eight of the species tested no mortality was observed at rates of 1440 to 7200 g a.i./ha in the range-finding test, but the EC_{50} values for dry weight in the definitive test for these same species were all less than 65 g a.i./ha—more than a 36 fold difference in rate between the 50% effect level for dry weight and the highest rate at which no mortality was observed.

Because of the internal inconsistencies in the Boutin et al. paper and the deviation from established test methods, the results from the regulatory studies should be utilized for risk assessment, rather than the results from the Boutin et al. study.

References (Review of Boutin et al., 2004)

Organization for Economic Co-operation and Development (OECD). 2006. OECD Guidelines for the Testing of Chemicals, Test No. 227: Terrestrial Plant Test: Vegetative Vigour Test. Available at: http://puck.sourceoecd.org/vl=4001279/cl=18/nw=1/rpsv/ij/oecdjournals/1607310x/v1n2/s27/p1

U.S. EPA. 1996. Ecological Effects Test Guidelines, OPPTS 850.4250 Vegetative Vigor, Tier II, Public Draft. Available at: http://www.epa.gov/ocspp/pubs/frs/home/draftguidelines.htm

U.S. EPA. 2008. Risks of Glyphosate Use to Federally Threatened California Red-legged Frog (*Rana aurora draytonii*). Pesticide Effects Determination. U.S. Environmental Protection Agency/Office of Pesticide Programs. Available at: http://www.epa.gov/espp/litstatus/effects/redleg-frog/.

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Author(s)	Year	Study title
Boutin, C., White, A. L., Carpenter, D.	2010	Measuring variability in phytotoxicity testing using crop and wild plant species Environmental Toxicology and Chemistry Volume: 29 Issue: 2 Pages: 327–337 Url: http://www.ncbi.nlm.nih.gov/pubmed/20821451 DOI: 10.1002/etc.30 ISSN: 0730-7268 (print), 1552-8618 (online)

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Abstract[82]

A series of experiments was conducted to assess the level of variability in phytotoxicity testing and to investigate factors that may explain some of the observed uncertainties and inconsistencies. The work was conducted in greenhouse or growth chamber environments with plants growing individually in pots and harvested 28 d after spraying with two herbicides, glyphosate and atrazine, as formulated products. Between six and 10 doses were used on five or six replicates, necessitating over 4,500 individually growing plants. In the first set of experiments, several ecotypes (originating from different areas of the world) of eight wild plant species were tested. Significant differences in sensitivity to atrazine and glyphosate were found among ecotypes of most species tested. In the second suite of experiments, the reproducibility of results during different seasons (when growing conditions vary) was investigated using three crops and four wild plant species. Results showed that seasonal variability elicited a pronounced discrepancy in response between plants tested at different times of the year. It was found that no consistent effects could be attributed to the biotic or abiotic factors investigated. Several ecotypes of the same species differed in their seed size, percentage germination, or germination requirements, as well as in growth patterns, but these differences could not explain differences in herbicide sensitivity. Likewise, differences in phytotoxicity could not be attributed to factors such as temperature, light intensity, and sunlight duration. The present study supports the inclusion of an uncertainty factor in risk assessments to account for the intrinsic variability in plant sensitivity to herbicides.

MATERIALS AND METHODS

1. Test material:

Test item:	Round-Up Original® or Vision®
Active substance(s):	Glyphosate
Surfactant:	Nonylphenoxy polyethyoxyethanol (Agral 90, Norac Concepts)
Description:	
Source of test substance:	Monsanto Canada
Lot/Batch #:	Not stated
Purity:	356g/L glyphosate [N-(phosphonomethyl) glycine]

2. Vehicle and/or positive control:

3. Test organism:

Species: <u>Ecotype sensitivity experiment</u>
Bellis perennis L.
Centaurea cyanus L.

[82] Quoted from article

Digitalis purpurea L.
Inula helenium L
Prunella vulgaris L.
Rumex crispus L.
Rudbeckia hirta L.
Solidago canadensis L.

Temporal variability experiment
Lycopus americanus Muhl.
Geum canadense Jacq.
Chrysanthemum leucanthemum L.
Triticum aestivum L.
Lactuca sativa L.
Solanum lycopersicon L.

Age of test organisms at study initiation: Three- to five-leaf stage

Source: Europe and North America, from commercial seed suppliers, donated or wild populations

Holding conditions prior to test: test with pesticides:
not specified
germination test
0- or 1-month stratification period coupled with two different environments (a greenhouse and a growth chamber, which exhibited differences in light intensity and temperature fluctuations)

4. Test system:

Study type: Greenhouse or growing chambers

Guideline: none

GLP: Not stated

Guideline deviations: -

Duration of study: 28 d

Test conditions: Plants were grown from seed and transplanted into 10-cm diameter by 9-cm-height plastic pots containing a 3:1 Promix BX with Mycorise1 Pro (Premier Horticulture) soil:sand mixture within 14 d of germination

Replicates per concentration: six (ecotype variability experiment)
six (temporal variability experiment)

Organisms per replicate: 1 plant per pot. Typically nontarget plant studies have multiple plants per replicate to reduce variability.

Parameters measured: Biomass (dry weight), germination (test without herbicide), seed size and number.

Test concentrations: Ecotype variability experiment
Nine or ten doses both following a geometric progression of 1.7 from 21 to 2,277 g a.i./ha

Temporal variability experiment:

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	Six to nine doses following a geometric progression ranging from 1.3 to 1.6 (53 to 2,285 g a.i./h)
Application:	6.75 ml/m^2 at 206.84 kPa
Application devices:	a track spray booth (de Vries Manufacturing) equipped with a TeeJet 8002E flat-fan nozzle (Spraying Systems)
Application verification:	not stated
Analytical determination of test concentrations:	not stated

5. Environmental conditions:

Test medium:	3:1 Promix BX with Mycorise1 Pro (Premier Horticulture) soil:sand mixture
Temperature:	16 – 43°C
Photoperiod:	16 h daylight
Light intensity:	106 – 1959 µmol m^{-2} s^{-1} (photosynthetically active radiation (PAR))
pH:	not stated

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment:

- Source of seeds are not precisely reported, only information about the country of origin are available
- Data on physical and chemical test conditions are missing
- No information about the verification of the application (e.g., measured concentration of glyphosate in spray solutions)
- No information about the watering regime and nutrient content of the soil
- ecotypes for the temporal variability experiment are not documented
- well documented: statistical methods/ results, effect concentrations

2. Relevance of study: **Relevant with restrictions**

Comment: Some useful information are missing (see above), but dose response curves are presented including effect concentrations (IC_{25} = 25% inhibition concentration), but no IC_{50} value which is the critical endpoint used in the EU non target plant risk assessments. IC_{25} values for biomass reduction on 14 species of plant for glyphosate ranged from 60 g a.s./ha to 1400 g a.s./ha in different tests and on different species. These endpoints are comparable to the range of IC_{25} values obtained in glyphosate data as submitted and summarized in section 8.12. The EC_{50} values would be higher.

Additional surfactant was added to the formulation, Nonylphenoxy polyethyoxyethanol, which is not known to be in commercial glyphosate-based formulation.

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As a non selective herbicide care should be taken to avoid spray drift when using this product. The lowest measured IC_{25} value for glyphosate in this study represents 2.8% of the maximum application rate for use in broadcast applications of 2160 kg a.s./ha. The standard model for drift exposure in the EU would predict the drift exposure level to be 2.77% of the application rate at 1m from the edge of the field, indicating that accidental spray drift from glyphosate application will represent a low risk to most species of plants and non target plant populations situated more that 1m from the sprayed target areas.

3. Klimisch code: **Klimisch rating of 2.**

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Author(s)	Year	Study title
Pfleeger, T., Olszyk, D., Lee, E.H., Plocher, M.	2011	Comparing effects of low levels of herbicides on greenhouse- and field-grown potatoes (*Solanum tuberosum* L.) soybeans (*Glycine max* L.) and peas (*Pisum sativum* L.). Environmental Toxicology and Chemistry. Volume: 30 Issue: 2 Pages: 455-468 DOI: 10.1002/etc.394 ISSN: 1552-8618 (online)

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Abstract[83]

Although laboratory toxicology tests are generally easy to perform, cost effective, and readily interpreted, they have been questioned for their environmental relevance. In contrast, field tests are considered realistic while producing results that are difficult to interpret and expensive to obtain. Toxicology tests were conducted on potatoes, peas, and soybeans grown in a native soil in pots in the greenhouse and were compared to plants grown outside under natural environmental conditions to determine toxicological differences between environments, whether different plant developmental stages were more sensitive to herbicides, and whether these species were good candidates for plant reproductive tests. The reproductive and vegetative endpoints of the greenhouse plants and field-grown plants were also compared. The herbicides bromoxynil, glyphosate, MCPA ([4-chloro-2-methylphenoxy] acetic acid), and sulfometuron-methyl were applied at below field application rates to potato plants at two developmental stages. Peas and soybeans were exposed to sulfometuron-methyl at similar rates at three developmental stages. The effective herbicide concentrations producing a 25% reduction in a given measure differed between experimental conditions but were generally within a single order of magnitude within a species, even though there were differences in plant morphology. This study demonstrated that potatoes, peas, and soybeans grown in pots in a greenhouse produce phytotoxicity results similar to those grown outside in pots; that reproductive endpoints in many cases were more sensitive than vegetative ones; and that potato and pea plants are reasonable candidates for asexual and sexual reproductive phytotoxicity tests, respectively. Plants grown in pots in a greenhouse and outside varied little in toxicity. However, extrapolating those toxicity results to native plant communities in the field is basically unknown and in need of research.

MATERIALS AND METHODS

1. Test material:

Test item:	Oust® (Sulfometuron-methyl), Roundup® (glyphosate), Buctril® (bromxynil), and Rhomene® (MCPA)
Active substance(s):	Oust®: methyl 2 [[[4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoate Roundup®: N-(phosphonomethyl)glycine Buctril®: 3,5-dibromo-4-hydroxybenzonitrile Rhomene®: [4-chloro-2-methylphenoxy]acetic acid
Surfactant:	Preference™ at 0.25% v/v with glyphosate (as well as with sulfometuron-methyl).
Source of test substance:	Not stated
Lot/Batch #:	Not stated

[83] Quoted from article

Purity: Not stated

2. Carrier: Water

3. Test organism:

Species: Potatoes (*Solanum tuberosum* L. cv. Russet Burbank) were tested with all 4 herbicides including glyphosate.

(In addition, soybeans (*Glycine max* L. cv. Stressland) and peas (*Pisum sativum* L. cv. Dakota) were tested with sulfometuron-methyl)

Source: Commercially purchased

Age of test organisms at study initiation: Potatoes: at tuber initiation 14 days after emergence (DAE) or at bulking 28 DAE.

Holding conditions prior to test: Test species seeds were planted in the greenhouse. After seedling emergence, plants for outside treatment were transferred to outdoor nursery.

4. Test system:

Study type: Greenhouse and potted field trials (outdoor nursery)

Guideline: U.S. Environmental Protection Agency (1996). Ecological effects test guidelines. OPPTS 850.4150 Terrestrial Plant Toxicity, Tier I (Vegetative Vigor). EPA 712-C-96-163. Washington, DC.

GLP: No

Guideline deviations:
- Potatoes used instead of suggested test species in guideline.
- Earlier application time does not follow guideline recommendations.

Duration of study: Appr. 4 months

Test conditions: Potatoes were grown in 19-L pots in the greenhouse and outside in the nursery. At 14 or 28 DAE plants were treated with herbicide. Plant height was evaluated prior to herbicide exposure and 14 DAE. Appr. 7 days prior to harvest, growth was stopped by withholding water. Harvest in greenhouse and nursery at the same time.

Water volume: 234 L/ha

Treatments: 28 treatments in total with a factorial design of 2 application times (tubing initiation and bulking) crossed with 2 locations (greenhouse and outside) and crossed with 5 herbicide rates and 1 water control and 1 non-spray control.

Replicates per treatment: 6

Individuals per replicate: 1 replicate = 1 pot = 1 plant

Parameters measured: Plant height (prior to and after herbicide application), shoot dry weight, tuber weight at harvest (size classified according to USDA guidelines[84])

Test concentrations: 0.46592, 2.6624, 14.976 and 83.2 g a.i./ha (corresponds to 0.00056, 0.00320, 0.01800 and 0.10000 of field application rate)

Application / device: RIC spray chamber (Model RC-500-100EP, Mandel Scientific) using a single even, flat, fan-spray nozzle (TeeJet spray nozzle

[84] United States Department of Agriculture (1983). United States standards for grades of potatoes for processing. Agricultural Marketing Service, Fruit and Vegetable Division, Fresh Products Branch. Washington, DC.

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	TP8002E-VS)
Verification of dispersion:	Delivery rate of the spray system was verified prior to each application.
Validity criteria:	None

5. Environmental conditions:

Test medium / Soil at study site:	Pasteurized Newberg sandy loam mineral soil
Weather conditions / Temperature / relative humidity:	Greenhouse:22.7°C (19.5-25.8°C), 67% Nursery: 18.7°C (9.9-27°C), 64%
Photosynthetically active radiation:	Greenhouse: 134 µmol m^{-2} sec^{-1} Nursery: 511 µmol m^{-2} sec
CO_2:	Greenhouse: 395 ppm Nursery: 403 ppm
Organic matter (C_{org}):	3%
Soil textural fractions / extractable micronutrient concentrations [mg per kg soil]:	Coarse-loamy, mixed, superactive, mesic Fluventic Haploxeroll. A deep, well-drained soil typical for the flood plains along the Willamette River (Oregon, USA).
Fertilization:	Scotts slow-release Osmocote fertilizer (14-14-14: N, P_2O_4, K_2O)

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- The use of one of the most critical abbreviations is confusing. DAE is assigned to mean "days after emergence" in first place in the material and methods" when herbicide application is described, but hereafter "14 DAE" is synonymous with "14 days after exposure" when measurements are described as well as in the results.
  Finally, it remains unclear due to uncertainties of the meaning of "DAE" whether plant height was measured at 14 days after exposure according to guidelines or at 14 days after emergence which would be on the day of the early application time.
- Withholding water at test termination (appr. 4 months after emergence) has significant impact on the measured parameters like shoot dry weight or tuber weight. In fact, growth was deliberately stopped by this way of test termination.

2. Relevance of study:

Comment: Measurement of plant height is unclear and the way of test termination questions the reliability.

Study does not meet the OECD guideline, spacing of rates is too large to calculate EC25.

Plant height should correlate with above ground biomass and does not and questions the reliability of this data. Fractional rate affecting plant height is 100 times greater than rate affecting above ground biomass (0.4 vs 0.004) for GH plants at TI. For application at bulking the fractional rate with a 25%

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effect on plant height (0.4) is comparable to that at tuber initiation but the 25% effect rate for above ground biomass at bulking is 0.04, 10 times less toxic than the 25% rate at tuber initiation.

No difference between above ground biomass between application times for outdoor grown plants which should be the more relevant exposure.

Fractional rate with 25% affect on mean tuber weight (0.03) is much higher than fractional rate affecting above ground biomass (0.004). Fractional rate with 25% effect on mean tuber weight between the two application timings for GH plants are comparable (0.03 vs 0.054). This seems unlikely if there was a significant difference in biomass.

The endpoint measured is of low relevance since yield, which is the most relevant endpoint is not affected at doses that low.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

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Author(s)	Year	Study title
Piotrowicz-Cieslak, A.I., Adomas, B., Michalczyk, D.J.	2010	Different glyphosate phytotoxicity of seeds and seedlings of selected plant species Polish Journal of Environmental Studies Volume: 19 Issue: 1 Pages: 123-129 Url: http://www.pjoes.com/abstracts/2010/Vol19/No01/15.html DOI: not stated ISSN: not stated

Abstract[85]

The aim of this study was to compare the physiological responses of six plant species (popular crops or plants recommended as indicators of soil pollution) to a wide range of glyphosate concentrations (0, 1, 3, 7, 10, 40, 80, 120, 180, 240, 400, 750, 1,000, 1,500, 1,700 and 2,000 µM). Percent germination, root length, seedling dry mass and *myo*-inositol content, as well as seedling leachate electroconductivity were determined in *Lepidium sativum, Sinapis alba, Sorghum saccharatum, Brassica napus, Lupinus luteus* and *Avena sativa*. Percent seed germination, seedling dry mass and electroconductivity of seedling leachates were not clearly affected by the herbicide and could not be used as indicators of its phytotoxicity. An metabolite induced by abiotic stresses in many plants, *myo*-Inositol, was very strongly stimulated by glyphosate at doses above 10 or 40 µM, depending on plant species. The sensitivity of analyzed plants to glyphosate, as manifested by root length, differed clearly. In *Avena sativa* the relationship between root length and glyphosate concentration was fairly linear over a wide range of herbicide doses (up to 240-400 µM). The most distinct drop in root growth at low herbicide doses was visible in *Sorghum saccharatum.* The results show that a mild stress affecting root length may not clearly modify seedling *myo*-inositol levels, that respond distinctly to stronger stresses. Not all indicator plants are equally suitable for analysis of biological activity of glyphosate residues. *Sorghum saccharatum* seems particularly sensitive.

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup-Ultra® 360 SL
Active substance(s):	Glyphosate IPA salt
Adjuvant:	Not stated
Description:	None
Source of test substance:	Not stated
Lot/Batch #:	Not stated
Purity:	360 g/L
2. Vehicle and/or positive control:	None

3. Test organism:

Species:	Oilseed rape (*Brassica napus*) White mustard (*Sinapis alba*) Yellow lupin (*Lupinus luteus*) Cress (*Lepidum sativum*) Oats (*Avena sativa*)

[85] Quoted from article

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Sorghum (*Sorghum saccharatum*)

Source: Not stated.

Crop growth stage at treatment: Seed

4. Test system:

Study type: Extended seedling emergence test

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 3-6 days

Test conditions: Seeds were germinated for three and six days using Phytotoxkit ™ (MicroBio Test Inc., Belgium) filled with 90 mL of reference soil under controlled climatic conditions (25 °C and 90% RH) in darkness. Soils were hydrated with 27 mL aqueous solutions and covered with filter paper.

Treatments: 15 test item treatments, 1 distilled water control.

Replicates per treatment: 9 for seed germination and root growth, 3-5 for *myo*-inositol

Seedlings per replicate: Not stated

Parameters measured: Germination was scored after 3 and 6 days, root length (image processing software), dry and fresh weight and electroconductivity after 3 and 6 days after imbibition. *Myo*-Inositol content of roots was determined by gas chromatography.

Results were evaluated statistically using ANOVA for two factor experiments, mean values were compared using Student-Newman-Keuls test.

Test concentrations: 1, 3, 7, 10, 40, 80, 120, 180, 240, 400, 750, 1000, 1500, 1700 and 2000 µM

5. Environmental conditions:

Test medium: Reference soil provided by Phytotoxkit ™ manufacturer containing sand, vermiculite and peat (1:0.3:1).

Water content: 27 mL

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment:

- Statistical analyses are not sufficiently described lacking selection criteria of statistical tests, data characteristics and parameter of test results (only the p-value is given). It does not allow to evaluation of result quality.
- No positive control.
- No information of content of adjuvants or surfactants.
- Not clear how doses used in this lab study transfer to field conditions, i.e. an application rate expressed as weight/ha. On p. 127 it is stated that 7 µM translates to 3.0 L Roundup Ultra 360 SL/ha. This implies a lowest rate tested of 154.3 g a.s./ha.

2. Relevance of study: **Not relevant**

Comment: Glyphosate does not have significant soil activity in natural soils, both because it is strongly bound and more importantly because it rapid degraded by soil micro-organisms; this has been demonstrated in a GLP study summarized in section IIA and is confirmed with the current study, using extremely high concentrations of glyphosate (up to 2000 µM, comparable to a field application rate of ~860 L Roundup/ha). Post emergence endpoints are within or above the range previously tested in GLP studies.

A point of principle - We have to assume that the "lab/petri dish" study was done in sterile conditions, and such a technique is not necessarily appropriate for monitoring contamination levels in real soils.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment**

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Author(s)	Year	Study title
Pline, W.A., Wilcut, J.W., Edmisten, K.L., Wells, R.	2002	Physiological and morphological response of glyphosate-resistant and non-glyphosate-resistant cotton seedlings to root-absorbed glyphosate. Pesticide Biochemistry and Physiology Volume: 72 Issue: 1 Pages: 48-58 Url: http://www.sciencedirect.com/science/article/pii/S0048357502000147 DOI: 10.1016/S0048-3575(02)00014-7 ISSN: 0048-3575

Abstract[86]

The level of tolerance in herbicide-resistant plants may vary among different tissues or growth stages. Studies were conducted to determine relative tissue sensitivity in glyphosate-resistant (GR) and non-GR cotton seedlings to the herbicide glyphosate. Glyphosate is often applied as a pre-plant treatment (burndown) in minimal tillage cotton production systems to remove any unwanted, emerged vegetation. Timing of these glyphosate applications may be in close proximity to the time of planting and seedling emergence. As glyphosate leaches from roots of nearby senescing weeds, it may be absorbed into the roots of cotton seedlings. Therefore, cotton seedlings were grown in hydroponic solutions containing technical grade glyphosate to ensure constant exposure to glyphosate. In all tissues, GR cotton required a greater concentration of glyphosate to reach 50% fresh weight reduction than non-GR cotton. Glyphosate inhibited the growth of non-GR cotton cotyledons, hypocotyls, and roots 50% at concentrations of 23, 69, and 27 µM glyphosate, respectively. In contrast, growth of GR cotton cotyledons, hypocotyls, and roots was inhibited by 50% at 3.5-, 8-, and 5-fold greater glyphosate concentrations, respectively, than non-GR cotton tissues. Correspondingly, shikimic acid, an intermediate in the shikimic acid pathway, which accumulates upon 5-enolpyruvyl 3-shikimate phosphate synthase (EPSP synthase) inhibition, reached levels of 17.3, 21.6, and 8.8 µM g^{-1} fresh weight at 1 mM glyphosate in non-GR cotyledons, hypocotyls, and roots, respectively. In contrast, shikimic acid levels in GR cotton were 4.2, 14.0, and 8.2 µM g^{-1} fresh weight at 1 mM glyphosate for cotyledons, hypocotyls, and roots, respectively. Thus, roots of GR and non-GR cotton accumulate similar amounts of shikimic acid, whereas GR cotyledons and hypocotyls accumulated less shikimic acid than the corresponding non-GR tissues in response to glyphosate treatments. Additionally, glyphosate inhibited the development of lateral roots at concentrations of 0.01 or 0.1 µM glyphosate greater, in GR and non-GR cotton, respectively. Lateral roots of GR and non-GR cotton inhibited by glyphosate appeared shorter and were surrounded by a thick layer of necrotic cells or root exudate which was not present in roots from plants grown in media not containing glyphosate. The quantity of GR CP4-EPSP synthase was 4.7 and 6.6 times greater in cotyledons than in hypocotyls and roots, respectively. Tissues from dark-grown GR cotton seedlings contained 1.2–2.1 times less CP4-EPSP synthase than their light-grown counterparts. Because lateral root development was inhibited, fresh weight was reduced, and shikimic acid accumulated following treatment with glyphosate in both GR and non-GR cotton, the potential exists for glyphosate to negatively affect cotton seedling establishment.

MATERIALS AND METHODS

1. Test material:

Test item(s):	Technical grade glyphosate
Active substance(s):	glyphosate
Adjuvant:	none

[86] Quoted from article

Description: none

Source of test substance: Sigma, St. Louis, MO, USA

Lot/Batch #: Not stated

Purity: 95%

Stock solution: Not stated

2. Vehicle and/or positive control: None

3. Test organism:

Species: Cotton (*Gossypium hirsutum* L.) - Glyphosate-resistant (GR) cultivar 5415RR and non-GR resistant cultivar 5415

Age of test organisms at application: 7-9 days, when cotyledons emerged from seed coat.

Holding conditions prior to test: Seeds were germinated for 1 week in petri dishes containing moist blotter paper in a dark growth chamber at 25 °C.

Acclimatisation: none

4. Test system:

Study type: Hydroponic lab study

Guideline: None

GLP: No

Duration of study: 10 days after application

Test conditions: Seedlings were hydroponically kept in scintillation vials containing 20 mL nutrient solution. Nutrient solutions were replaced every 3 days. After 10 days, plants were separated into cotyledons/first true leaf, hypocotyl and radical/lateral roots.

Treatments: 4 glyphosate concentrations and one glyphosate untreated control.

Replicates per treatment: 3

Individuals per replicate: 4

Parameters measured: Fresh weight of each tissue after 10 days of application, visual rating of lateral root formation. Amount of shikimic acid in cotyledons, hypocotyl and roots at 380 nm. Quantification of CP4-EPSP synthase (5415RR plants only).

Lateral root development by image analysis of fixated root particles of plants exposed to 0 and 0.1 mM glyphosate (GR seedlings) and to 0 and 0.001 mM glyphosate (non-GR seedlings)

Test concentrations: 0, 0.1, 1, 10 and 100 µM glyphosate (corresponding to 0, 0.169, 1.69, 16.9 and 169.1 ppm)

Analytical determination of test concentrations: None

Validity criteria: None

5. Environmental conditions:

Test medium: Hydroponic nutrient solution containing 500 mg/L Peters 20-20-20 fertilizer (J.R. Peters, Allentown, PA)

pH: Not stated

Temperature: Not stated

Photoperiod: Not stated

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Lighting Not stated

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment

- Weibull distribution as statistical tool for evaluation of growth response (fresh weight) is not appropriate; further stats seem to contain a mix of parameters based on measured and subjective observations.
- Rationale not provided for how concentrations tested translate to field relevant concentrations.
- Source of test organisms are not reported
- Omissions in reporting of experimental detail do not allow a reader to distinguish effects from glyphosate and pH or other abiotic factors.

2. Relevance of study: **Not relevant**

Comment: Unclear how these results translate to more ‘natural’ conditions, i.e. using natural plant growth substrate such as soil. The application of herbicides on hydroponic growth cultures is extremely unrealistic. Additionally, results from a GLP study summarized in section IIA 8.12 evaluating glyphosate for pre-emergence activity demonstrates that glyphosate does not have pre-emergence activity at relevant field exposure levels.

3. Klimisch code: **Klimisch rating of 3 and not acceptable for risk assessment.**

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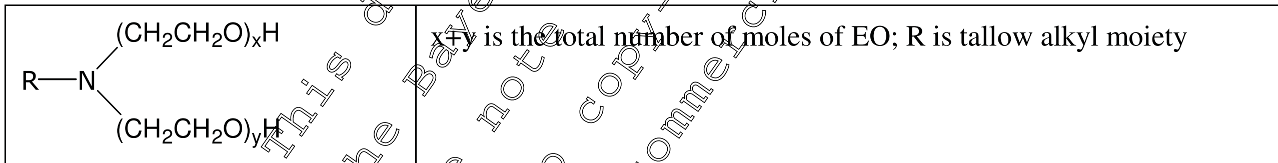
Overview of polyethoxylated tallow amine (POEA)

Polyethoxylated tallow amine (POEA) is a non-specific name which covers a range of alkylamine ethoxylate compounds. No specific CAS no. has been allocated because this is a large and varied group of chemistry. POEA is also sometimes used to refer to an even broader range of compounds, the polyoxyethyleneamines of which the polyoxyethylenealkylamines are a sub-group.

POEA surfactants are nonionic and are more widely used as detergents over ionic surfactants because anionic surfactants are insoluble in many hard water and cationic surfactants are considered to be poor cleaners. In addition to detergency, nonionic surfactants show excellent solvency, low foam properties and chemical stability. POEA is structurally comparable to alcohol ethoxylates which are commonly used in household products as hard surface cleaners (e.g., floors) and for home dish washing. **The same properties that allow these detergents to remove grease from dishes and pots and pans are the same properties that allow them to penetrate the waxy cuticle of leaves. Consequently, we are exposed to surfactants and particularly nonionic surfactants every time we do our dishes and even when we wash our bodies to remove natural oils. Levine et al. 2007 demonstrates the comparable properties of surfactants and their comparable properties on cell membranes.**

Polyethoxylated tallow amine surfactants are used with a wide range of pesticidal products, either as a co-formulant or as tank mix adjuvant. Amine ethoxylate products are used as acid thickeners, emulsifiers, dispersants, antistatic agents, textile softeners and lubricants, and as wetting agents in both acid and alkaline solutions. As corrosion inhibitors, they are used in aqueous acidic solutions, refined petroleum products, and formulations for use in the production and refining of petroleum. In addition, amine ethoxylates are applied as components in mill-processing formulations for fabrication of metal and textile products, as wetting agents for asphalt, and as frothing agents for ore flotation (Encyclopedia of Chemical Technology, 4th edition, John Wiley & Sons).

Polyethoxylated tallow amine, also referred to as polyoxyethylene tallow amine, covers a class of non-ionic surfactants containing tallow amines which have been ethoxylated, or reacted with ethylene oxide, to obtain a certain degree of ethoxylation, resulting in a series of compounds:



A comprehensive summary of the ecotoxicological data for the MON 0818 (a code designation for the preparation of POEA used in Monsanto formulations) and a formulation containing a maximum loading of POEA is presented in Giesy et al. 2000. This article is a suggested source for wildlife effects information and associated references.

References

Levine SL, Han Z, Liu J, Farmer DR, Papadopoulos V. (2007). Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. Cell Biol Toxicol. 2007 Nov;23(6):385-400.

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Exposure and Effects of POEA to Aquatic Animals

Aquatic organisms may be exposed to POEA surfactants as a consequence of the unintended entry of an herbicide formulation into the aquatic compartments via spray drift or as a consequence of run-off and drainage events. The EXPOSIT surface water model was used to estimate maximum predicted environmental concentrations of POEA in surface water (PEC_{sw}). The estimates are made for applications corresponding to 780 g POEA/ha, which is believed to address the maximum application rate for broadcast applications of POEA[87]. A summary of the input parameters used in the surface water modeling is provided in Table 1. These input parameters are from studies conducted with radiolabeled G3780 (now referred to as MON 0818). Based on these values POEA is categorised as Category I substance in Exposit although the degradation rate is faster and the solubility higher than indicated in Category I.

Table 1. Input parameters for surface water modeling of POEA.

Parameter	Value
Water Solubility	**>382 mg/L** [a]
K_{oc}	**2500** - 9600 [b]
DT_{50} Soil	7-**14** days [b]
DT_{50} Water /Sediment System	21-**42** days [b]

[a] USEPA 2009

[b] Values as cited in Giesy et al. 2000. These values are based on environmental fate studies reported in Marvel et al. 1974 with some conservatism added. The National Regulatory Authority of Australia (1996) reviewed the Marvel study and concluded the soil DT_{50} value was ≤ 7 days and the water /sediment DT_{50} value was 14 days. Values used for Exposit modeling are bolded.

It is important to note that POEA surfactants do not contain molecules of a single molecular structure, but rather these surfactants are a complex mixture of molecules with different aliphatic chain lengths and different numbers of ethylene oxide units. The environmental fate endpoints of MON 0818, therefore, reflect the overall trend in sorption and degradation kinetics of the various molecular components in the surfactant mixture.

Biodegradation of POEA surfactants was investigated by Van Ginkel et al. (1993). They report that biodegradation consists of two phases, a central fission at the nitrogen atom with rapid mineralization via oxidation of the alkyl chain, followed by degradation of the ethoxylated secondary amine (Van Ginkel et al., 1993). The toxicity of these two intermediates to aquatic vertebrates is predicted to be significantly lower than that of the parent molecule. The ethoxylated secondary amine is structurally very similar to polyethylene glycol, which has generally very low aquatic toxicity (Van Ginkel et al., 1993; WHO, 2000, Sec. 7.1, p. 11). The alkyl chain intermediate is predicted to have low toxicity to aquatic vertebrates as well (USEPA, 1992, p. 13).

A more recent study examined the dissipation of POEA in a water sediment system (Wang et al., 2005). In this study, the POEA surfactant MON 0818 was prepared at a target concentration of 8 mg/L in well water. Two natural sediments (1.5% and 3.0 % organic matter) were layered into triplicate separate glass aquaria (80 x 30 x 30 cm) to a depth of 3 cm and then 12 cm of the MON 0818-containing water was added to the sediments or to empty aquaria. Well water alone was also added to each of the sediments as another treatment in the study. The removal of MON 0818 from the water column was monitored over time using both a biological assay and chemical analysis. Water samples were removed from each aquarium at 2, 6, 24, 48, 72, and 96 hours. The samples were analyzed using a toxicity bioassay with Daphnia magna and using high performance liquid chromatography with mass selective detection for

[87] Specific details about the amount of surfactant per liter formulation can not be discussed in the shared Industry document for ani-trust reasons. For this calculation is was asummed that the maximum application rate is 2.16 kg a.s./ha and the the POEA content is approximately 36% of the glyphosate acid content.

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intact surfactant. The authors reported, "The toxicity of the POEA surfactant, MON 0818, decreased rapidly in water from microcosms containing sediment, and this decrease in toxicity was correlated with the decline of MON 0818 concentrations in the overlying water." Dissipation rates could be calculated from the analytical measurements of POEA over time, and DT50 values for the removal of POEA from the water column in the presence of the two sediments were calculated to be 18 hrs and 13 hrs, respectively. It is likely that both physical adsorption to the sediment and microbial degradation played some role in the rapid reduction in toxicity and surfactant removal from the water column. However, since the instrumental analysis only focused on the molecular ions for the intact surfactant molecules, no estimate of the contribution of microbial degradation to the dissipation rate can be made from this study.

The PEC_{sw} values considered in the TER_A calculations are included in the tables in the following sections. A summary of the maximum PEC_{sw} values from drift obtained from the EVA 2.0 calculations is in Table 2 and for the EXPOSIT 2.0b drainage and run-off modelling is provided in Table 3 and 4. EXPOSIT 2.0b is used for the modeling as the Koc is > 500.

Table 2: Predicted environmental concentrations of POEA in surface water (PEC_{sw}) due to drift (EVA)

Distance (m)	Drift * (%)	780 g POEA/ha PEC (µg POEA/l)
0	100	260
1	2.77	7.2
5	0.57	1.5
10	0.29	0.78

* Rautmann's drift values [88]

Table 3: Predicted environmental concentrations of POEA in surface water (PEC_{sw}) due to run-off calculated with Exposit 2.0b:

Distance (m)	780 g POEA/ha PEC(µg POEA/l)
0	3.13
5	1.73
10	0.378

Table 4: Predicted environmental concentrations of POEA in surface water (PECsw) due to drainage calculated with Exposit 2.0b:

Distance (m)	Timing	780 g POEA/ha PEC (µg POEA/l)
0	Spring	0.108
0	Autumn	0.324

The PEC_{SW} values from drainage in Tables 4 can be considered worst-case concentrations, as they represent the predicted global maximum concentrations. Sediment concentrations are not considered in this assessment, so the values are not reported.

Measurements of the dissipation of POEA from the water column in two water sediment systems have demonstrated that POEA is rapidly removed from the water column with dissipation half-lives of less than 1 day (Wang et al. 2005). It is therefore unlikely that chronic exposure to POEA will occur in the water column. Time-weighted average concentrations are reported in Table 5 for the highest initial concentration originating from runoff assuming a DT_{50} of 1 day.

[88] Rautmann, D; Streloke, M., Winler, R. (2001): New basic drift values in the authorisation procedure for plant protection products. In Forster, R.; Streloke, M. Workshop on Risk Assessment and Risk Mitigation Measures in the Context of the Authorization of Plant Protection Products (WORMM). Mitt.Biol.Bundesanst.Land- Forstwirtsch. Berlin-Dahlem, Heft 381.

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Table 5. Predicted time-weighted average environmental concentrations of POEA in surface water (PEC_{sw}) due to runoff calculated with Exposit 2.0b

	PEC actual (µg POEA/l)	PEC_{twa} [a] (µg POEA/l)
Days after peak concentration	3.132	3.132
1	1.566	2.262
2	0.786	1.692
4	0.198	1.062
7	0.024	0.642
14	0.000	0.324
21	0.000	0.216
28	0.000	0.162
42	0.000	0.108

[a]Time-weighted averages calculated assuming a 50% dissipation of POEA from the water column in 1 day.

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Effects on aquatic organisms

Acute toxicity data is available for the POEA containing surfactant MON 0818 for three invertebrate species, nine fish species, and two amphibian species (Giesy et al. 2000). MON 0818 has been reported to contain 75% POEA (Wan et al. 1989) or 69-73% POEA (Howe et al. 2004). Table 6 provides the acute toxicity data for MON 0818 giving toxicity values as both MON 0818 and as POEA. LC_{50} / EC_{50} values for all 13 species range from 0.49 – 9.8 mg (490 – 9800 µg) POEA/L.

Table 6. Acute Aquatic Toxicity Data for MON 0818 (75% POEA Surfactant)

Species	Test Duration (days)	EC50 or LC50 (mg MON 0818/L)	Corrected EC/LC50[a] (mg POEA/L)	Reference
Invertebrates				
Chironomus plumosus	2	13	9.8	Folmar et al.,1979
Daphnia magna	2	2	1.5	ABC Inc., 1980a
Daphnia pulex	2	4.1	3.1	Moore et al., 1987
Daphnia pulex	4	2	1.5	Servizi et al., 1987
Fish				
Bluegill sunfish (*Lepomis macrochirus*)	4	1.3	1.0	ABC Inc., 1980b
Bluegill sunfish (*Lepomis macrochirus*)	4	1.0 - 3.0	0.8-2.3	Folmar et al., 1979
Channel catfish (*Ictalurus punctatus*)	4	13	9.8	Folmar et al., 1979
Chinook salmon (*Oncorhynchus tshawytsha*)	4	2.8, 1.7[b]	2.1, 1.3[b]	Wan et al., 1989
Chum salmon (*Oncorhynchus keta*)	4	2.4, 1.4[b]	1.8, 1.1[b]	Wan et al., 1989
Coho salmon (*Oncorhynchus kisutch*)	4	3.2, 1.8[b]	2.4, 1.4[b]	Wan et al., 1989
Coho salmon fry (*Oncorhynchus kisutch*)	4	3.5	2.6	Servizi et al., 1987
Fathead minnow (*Pimephales promelas*)	4	1	0.8	Folmar et al., 1979
Pink salmon (*Oncorhynchus gurbuscha*)	4	2.8, 1.4[b]	2.1, 1.1[b]	Wan et al., 1989
Rainbow trout (*Oncorhynchus mykiss*)	4	4.2	3.2	ABC Inc.,Inc. 1980c
Rainbow trout	4	0.65 - 7.4	0.49 – 5.6	Folmar et al., 1979
Rainbow trout fry	4	3.2	2.4	Servizi et al., 1987
Rainbow trout	4	2.5, 1.7[b]	1.9, 1.3[b]	Wan et al., 1989
Sockeye salmon fry (*Oncorhynchus nerka*)	4	2.6	2.0	Servizi et al., 1987

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Amphibians				
Xenopus laevis embryos	4	6.8	4.8	Perkins et al., 2000
Xenopus laevis embryos -larvae	4	5.0	3.5	Perkins 1997.
Xenopus laevis Gosner Stage 8-10 pH 8.0	4	1.4 – 1.5	0.98 – 1.1	Edginton et al. 2004
Xenopus laevis Gosner Stage 8-10 pH 6.5	4	3.0 – 3.9	2.1 – 2.7 [c]	Edginton et al. 2004
Rana clamitans Gosner Stage 25	4	1.1	0.77[c]	Howe et al., 2004

[a] Converted from mg MON 0818/L to mg POEA/L assuming MON 0818 is 75% POEA by weight (Wan et al. 1989).
[b] Soft and hard water values, respectively.
[c] Converted from mg MON 0818/L to mg POEA/L assuming MON 0818 is 70% POEA by weight (Howe et al., 2004).

Long-term toxicity data for Roundup has been used to predict the long-term toxicity for POEA (Table 7) using the assumption that toxicity that is observed to fish or aquatic invertebrates from exposure to glyphosate formulations containing POEA is almost exclusively due to the POEA rather than the glyphosate present in the formulation.

Table 7. Long-term Aquatic Toxicity Data for POEA extrapolated from Roundup Effects Data

Species	Test Duration (days)	NOEC (mg Roundup/L)	Corrected NOEC[a] (mg POEA/L)	Reference
Invertebrates				
Daphnia magna	21	3.2	0.36	ABC Inc., 1989a
Fish				
Rainbow trout	21	2.4	0.27	ABC Inc., 1989b

a Conversion from Roundup to POEA assumes the Roundup formulation contains 11.25% POEA (Giesy et al. 2000 and Wan et al. 1989). Toxicity exposure ratios for aquatic species

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TER_A for fish and aquatic invertebrates

Acute toxicity exposure ratios (TER_A) for fish and aquatic invertebrates were calculated using toxicity endpoints for MON 0818 corrected for POEA content (see Table 8).

Table 8. Acute TER (TER_A) values for fish and aquatic invertebrates exposed to POEA at an expected maximum application rate (780 g POEA/ha), based on EVA and EXPOSIT 2.0b modelling with a 5 m buffer.

Test material: Species	96-hr LC_{50} (mg POEA/L)[1]	Directive 91/414/EC Annex VI trigger value	Maximum PECsw (mg a.s./L) [2]		TER_A [3]	
			Drift	Run-off	Drift	Run-off
MON 0818 exposure (corrected for POEA content)						
Oncorhynhus mykiss rainbow trout	0.49	100	0.00150	0.00173	**327**	**283**
Lepomis macrochirus bluegill sunfish	0.80	100	0.00150	0.00173	**533**	**462**
Daphnia magna	1.5	100	0.00150	0.00173	**1000**	**867**

Bolded values indicate TER is greater than or equal to the trigger value.
[1] From Table 6.
[2] From Table 2 and 3, assuming a 5 meter buffer zone
[3] $TER_A = LC_{50}/PECsw$, rounded to no more than 3 significant figures.

The TER_A values presented in Table 7 are above the Directive 91/414/EC Annex VI trigger value of 100 when POEA acute toxicity data are considered. If TER values were calculated for amphibians, those values would also be greater than the trigger value of 100. Since these values were calculated using the initial PEC, they may be considered extremely conservative. Since initial PEC values are much lower for drainage than for drift and run-off, TER values for drainage will also be above the Annex VI triggers (TER values not shown). Therefore it may be concluded that **acute risk for fish, amphibians, and aquatic invertebrates from exposure to POEA will be low**.

TER_{LT} for fish

Chronic toxicity studies are not available with POEA directly; however, a 21-day fish study and a *Daphnia* life-cycle study are available for a Roundup formulation. POEA exposure levels from this glyphosate formulation have been calculated to assess the long-term risk of POEA assuming all toxicity is due to the POEA. As recommended in the Guidance Document on Aquatic Ecotoxicology, the initial predicted environmental concentrations should be employed in the initial calculation of the long-term toxicity exposure ratio (TER_{LT}). The EVA and Exposit models≥ maximum PEC values for surface water, as calculated for the acute assessment, were considered. TER_{LT} values for fish and aquatic invertebrates are shown in Table 9.

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Table 9. Long-term TER (TER_{LT}) values for fish exposed to POEA at an expected maximum application rate (780 g POEA/ha), based on EVA calculations and EXPOSIT modelling with a 5 m buffer.

Test material: Species	NOEC (mg a.s./L)[1]	Directive 91/414/EC Annex VI trigger value	Maximum PECsw (mg POEA/L)[2]		TER_{LT}[3]	
			Drift	Runoff	Drift	Runoff
POEA calculated from Roundup NOEC values						
Oncorhynchus mykiss, 21-day study	0.36	10	0.00150	0.00173	**240**	**208**
Daphnia magna 21-day	0.27	10	0.00150	0.00173	**180**	**156**

Bolded values indicate TER is greater than or equal to the trigger value.
[1] From Table 4).
[2] From Tables 2 and 3(maximum PEC values with a 5 meter buffer).
[3] TER_{LT}= NOEC / PECsw; rounded to 3 significant figures.

The calculations show that the long-term TER values for fish and *Daphnia* are above the Directive 91/414 Annex VI trigger of 10. Since these values were calculated using the initial PEC values, they may be considered extremely conservative. Furthermore, water sediment studies have shown that the dissipation of POEA from the water column is much faster than predicted based on degradation with a half-life in the water column of less than 1 day (Wang et al., 2005) due to the high K_{oc}. Therefore, it may be concluded that **chronic risk for fish and aquatic invertebrates from exposure to POEA will be low**.

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Assessment of Synergy between Glyphosate and Surfactant

The concept of simple joint action, also referred to as concentration addition, was used to evaluate the joint action of glyphosate and POEA in a mixture (Roundup) to aquatic organisms. This assessment was based primarily on fish data from Wan et al. (1989) and Folmar et al. (1979), but data from Perkins et al. (2000), Folmar et al. (1979) and Tsui and Chu (2003) was used for amphibians and aquatic invertebrates.

From these sources, the joint action of glyphosate and POEA was examined using the concept of relative potency in order to assess potential interactions (antagonism, synergism) among components within a mixture (Durkin 1981). For example, the toxicity of a mixture of glyphosate and POEA (Roundup) was predicted from the relative potency (ρ) of the components

$$\rho = \frac{LC50a\,(GLY_{IPA})}{LC50b\,(POEA)}$$

Where appropriate, glyphosate LC50 values reported as mg acid equivalent (a.e.)/L need to be corrected to mg glyphosate IPA salt/L (mg a.e./L ÷ 0.74) prior to determining the relative potency to allow for more direct predictions of Roundup toxicity.

The toxicity of the mixture (Roundup) relative to the toxicity of the GLY_{IPA} (LC50a) should be estimated based on the relative proportions of GLY_{IPA} (pa) and POEA (pb) in the mixture (mg Roundup/L). Where the proportions of (a) and (b) are 41% and 11.25% of the formulation, respectively. (The Roundup formulation under consideration contains 15% MON 0818 which is a surfactant blend that is 75% POEA and 25% components with low toxicity (Wan et al., 1989; Wang et al., 2005). The proportion of POEA is therefore 15% x 0.75 or 11.25%). When surfactant LC50 values are expressed in MON 0818 units, these values need to be converted to POEA units based on the fraction of POEA in the surfactant MON 0818 (MON 0818 × 0.75) (See Wan et al. (1989) Tables 1 and 4). The toxicity data (LC50 values) for glyphosate technical material, glyphosate isopropylamine salt, and Roundup may be reported in units of mg a.e./L and should be converted to mg Glyphosate IPA/L for glyphosate or glyphosate IPA salt LC50 values and to mg formulation/L for Roundup LC50 values prior to the calculations described in this appendix.

$$pmix = pa + (\rho \times pb)$$

The predicted LC50 of Roundup (mg formulation/L) is estimated using

$$LC50\,mix = \frac{LC50a}{pmix}$$

Based on the above model assumptions, the predicted LC50 concentrations of Roundup formulation (mg formulation/L) appear to have been calculated incorrectly. A sample calculation using Wan et al. (1989) data for the Coho Salmon is illustrated below.

$$GLY_{IPA} = \frac{GLY}{0.74} = \frac{27}{0.74} = 36.5$$

$$\rho = \frac{LC50a\,(GLY_{IPA})}{LC50b\,(POEA)} = \frac{36.5}{4.6} = 7.93$$

$$pmix = 0.41 + (7.93 \times 0.1125) = 1.30$$

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$$LC50_{Roundup} = \frac{36.5}{1.30} = 28.0\ mg/L$$

This estimated toxicity of the mixture is then compared with the observed toxicity of the mixture. Ratios >1 suggest some form of greater than additive toxicity, and, conversely, ratios <1 indicate less than additive toxicity.

$$\frac{28\ mg\ Roundup/L}{32\ mg\ Roundup/L} = 0.88$$

Table 1 contains joint action calculations for fish species based on data from the Wan et al. (1989) paper. Calculations shown above vary from those presented in the SERA assessment because it appears that surfactant LC50 values in the Wan et al. article were corrected for the amount of POEA in MON 0818 (See Wan et al. Table 1 and footnote to Table 4). The proportion of the mixture that is POEA should, therefore, be 11.25% rather than 15%. The glyphosate toxicity data for Wan et al. (1989) are reported in units of mg a.e./L and should be converted to mg Glyphosate IPA/L. Roundup LC50 values are reported in formulation units in the paper and do not require conversion.

Table 2 contains the calculations for fish species from the Folmar et al (1979) paper. For this paper, surfactant LC50 values are expressed as MON 0818 and require a conversion to POEA (MON 0818 LC50 x 0.75). The glyphosate toxicity data for both technical material and Roundup in Folmar et al. are reported in units of mg a.e./L and must be converted to mg Glyphosate IPA/L and mg Roundup formulation/L, respectively. Based on the stated content of glyphosate in Roundup (360.32 mg/L) in the Folmar et al. paper and assuming a density of 1.172 g/cm^3, glyphosate would comprise 30.7% of the formulation by weight. This value is used as the conversion factor between Roundup formulation LC50 values expressed as glyphosate acid equivalents and as formulation units.

Table 3 contains the calculations for aquatic invertebrates from the Folmar et al. (1979) paper and from the Tsui and Chu (2003) paper. For the data from the Folmar et al. paper, the conversions described for Table 2 were also utilized for the aquatic invertebrate calculations. For the Tsui and Chu paper, the surfactant LC50 values were considered to be expressed as mg POEA/L. LC50 values for glyphosate acid, glyphosate IPA salt, and Roundup were all expressed as mg a.e./L and required conversion to mg gly IPA/L and mg formulation/L.

Table 4 contains the calculations for an amphibian species from Perkins et al. (2000). The surfactant LC50 was considered to be expressed as mg MON 0818/L and required conversion to mg POEA/L. LC50 values for Rodeo (glyphosate isopropylamine salt) and Roundup were both expressed as mg a.e./L and required conversion to mg glyphosate IPA salt/L and mg Roundup/L, respectively.

Based on the calculations presented in Tables 1 through 4, a conclusion of synergistic effects associated with glyphosate and POEA is not supported. The predicted to observed LC50 values for Roundup do not deviate markedly from unity, suggesting that no substantial interactions take place between these two compounds. In almost every case, the predicted and observed toxicity are within a factor of 2 suggesting that no substantial interactions take place between glyphosate and POEA (i.e., joint action is additive) (Belden et al., 2007).

The prediction of synergy in the SERA assessment is also in direct contrast to previous work examining the joint action of glyphosate and POEA (Diamond and Durkin, 1997).

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Table 1. Joint Action Calculations for Salmonids from Wan et al. (1989).

Wan Fish Species	pH	Component LC50 GLY (a.e./L)	GLY IPA (mg/L)	POEA (mg/L)	Potency	Roundup Observed (mg form./L)	Predicted (mg form./L)	Predicted/ Observed
Coho	6.3	27	36.5	4.6	7.93	32	28.0	0.88
	7.2	36	48.6	3.2	15.20	27	22.9	0.85
	7.8	112	151.4	2.8	54.05	33	23.3	0.71
	7.8	111	150.0	2.9	51.72	30	24.1	0.80
	8.2	174	235.1	1.8	130.63	13	15.6	1.20
Chum	6.3	10	13.5	2.7	5.01	20	13.9	0.69
	7.2	22	29.7	2.4	12.39	19	16.5	0.87
	7.8	99	133.8	2.6	51.46	15	21.6	1.44
	8.2	148	200.0	1.4	142.86	11	12.1	1.10
Chinook	6.3	19	25.7	2.8	9.17	33	17.8	0.54
	7.2	30	40.5	2.8	14.48	27	19.9	0.74
	7.8	102	137.8	2.7	51.05	19	22.4	1.18
	7.8	108	145.9	2.6	56.13	22	21.7	0.99
	8.2	211	285.1	1.7	167.73	17	14.8	0.87
Pink	6.3	14	18.9	4.5	4.20	33	21.4	0.65
	7.2	23	31.1	2.8	11.10	31	18.7	0.60
	7.8	94	127.0	1.5	84.68	17	12.8	0.75
	7.8	102	137.8	2.6	53.01	19	21.6	1.14
	8.2	190	256.8	1.4	183.40	14	12.2	0.87
Rainbow	6.3	10	13.5	2	6.76	33	11.5	0.35
	7.2	22	29.7	2.5	11.89	15	17.0	1.13
	7.8	99	133.8	1.6	83.61	18	13.6	0.76
	7.8	93	125.7	2.6	48.34	18	21.5	1.19
	8.2	197	266.2	1.7	156.60	14	14.8	1.05

Glyphosate LC50 values expressed as a.e./L were converted to GLY IPA mg/L (GLY ÷ 0.74 = GLY IPA).

Table 2. Joint Action Calculations for Fish from Folmar et al. (1979).

Folmar Fish Species	pH	Component LC50 GLY (a.e./L)	GLY IPA (mg/L)	MON 0818 (mg/L)	POEA (mg/L)	Potency	Roundup Observed (mg a.e./L)	Observed (mg form./L)	Predicted (mg form./L)	Predicted/ Observed
Rainbow Trout	6.5	140	189.2	7.4	5.55	34.09	7.6	24.8	44.6	1.80
	9.5	240	324.3	0.65	0.4875	665.28	1.4	4.6	4.3	0.95
Bluegill	6.5	140	189.2	1.3	0.975	194.04	4.2	13.7	8.5	0.62
	9.5	220	297.3	1.0	0.75	396.40	1.8	5.9	6.6	1.13
Rainbow Trout		140	189.2	2.0	1.5	126.13	8.3	27.0	13.0	0.48
Fathead Minnow		97	131.1	1.0	0.75	174.77	2.3	7.5	6.5	0.87
Channel Catfish		130	175.7	13	9.75	18.02	13	42.3	72.1	1.70
Bluegil		140	189.2	3.0	2.25	84.08	5.0	16.3	19.2	1.18

Glyphosate LC50 values expressed as mg a.e./L were converted to GLY IPA mg/L (GLY ÷ 0.74 = GLY IPA).
Surfactant LC50 values expressed as mg MON 0818/L were converted to mg POEA/L (MON 0818 x 0.75 = POEA)
Roundup LC50 values expressed as mg a.e./L were converted to mg RU/L using a conversion factor of 0.307 based on information in the Folmar paper. (mg a.e./L ÷ 0.307 = mg RU/L)

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Table 3. Joint Action Calculations for Aquatic Invertebrates from Folmar et al. (1979) and Tsui and Chu (2003).

Aquatic Invertebrates	Component LC50				Potency	Roundup			Predicted/ Observed
	GLY (a.e./L)	GLY IPA (mg/L)	MON 0818 (mg/L)	POEA (mg/L)		Observed (mg a.e./L)	Observed (mg form./L)	Predicted (mg form./L)	
Folmar									
Chironomus riparius	55	74.32	13	9.75	7.62	18	58.6	58.6	1.00
Tsui and Chu									
Ceriodaphnia dubia (GLY)	147	198.65		1.15	172.74	5.39	17.8	10.0	0.56
Acartia tonsa (GLY)	35.3	47.70		0.57	83.69	1.77	5.8	4.9	0.83
Ceriodaphnia dubia (GLY IPA)	415	560.81		1.15	487.66	5.39	17.8	10.1	0.57
Acartia tonsa (GLY IPA)	49.3	66.62		0.57	116.88	1.77	5.8	4.9	0.84

Glyphosate LC50 values expressed as mg a.e./L were converted to GLY IPA mg/L (GLY ÷ 0.74 = GLY IPA).
Surfactant LC50 values expressed as mg MON 0818/L were converted to mg POEA/L (MON 0818 x 0.75 = POEA)
Roundup LC50 values expressed as mg a.e./L in the Folmar et al. article were converted to mg RU/L using a conversion factor of 0.307 for *Chironomus riparius* based on information in the Folmar paper. (mg a.e./L ÷ 0.307 = mg RU/L).
Roundup LC50 values expressed as mg a.e./L in the Tsui and Chu paper were converted to mg RU/L using a conversion factor of 0.3034 (0.41 x 0.74). LC50 values were converted as follows: mg a.e./L ÷ 0.3034 = mg RU/L.

Table 4. Joint Action Calculations for Amphibians from Perkins et al. (2000)

Perkins Amphibians	Component LC50				Potency	Roundup			Predicted/ Observed
	GLY (a.e./L)	GLY IPA (mg/L)	MON 0818 (mg/L)	POEA (mg/L)		Observed (mg a.e./L)	Observed (mg form./L)	Predicted (mg form./L)	
Xenopus laevis	7296.8	9860.5	6.8	5.1	1933.44	9.3	30.65	45.2	1.48

Glyphosate LC50 values expressed as mg a.e./L were converted to GLY IPA mg/L (GLY ÷ 0.74 = GLY IPA).
Surfactant LC50 values expressed as mg MON 0818/L were converted to mg POEA/L (MON 0818 x 0.75 = POEA)
Roundup LC50 values expressed as mg a.e./L in the Perkins paper were converted to mg RU/L using a conversion factor of 0.3034 (0.41 x 0.74). LC50 values were converted as follows: mg a.e./L ÷ 0.3034 = mg RU/L.

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Glyphosate Endocrine Assessment

Introduction

In June 2007, EPA published in the Federal Register a Notice announcing the *Draft List of Initial Pesticide Active Ingredients and Pesticide Inerts to be Considered for Screening under the Federal Food, Drug, and Cosmetic Act* [72 FR 33486]. The initial chemicals to be tested under the EPA Endocrine Disruptor Testing Program were selected based on four human exposure pathways described in detail in an earlier Federal Register notice, namely, food consumption, drinking water consumption, residential use exposure, and occupational exposure (contact with pesticide-treated surfaces) [70 FR 56449]. The highest priority for inclusion on the initial list was for substances having potential exposure through all four pathways, which glyphosate does. Throughout the selection process, EPA clearly stated that "*[t]his list should not be construed as a list of known or likely endocrine disruptors. Nothing in the approach for generating the initial list provides a basis to infer that by simply being on the list these chemical are suspected to interfere with the endocrine systems of human or other species, and it would be inappropriate to do so.*" Presently, the Tier 1 screening and weight of evidence evaluation required by the USEPA is being completed. However, some of the results of this work is publically available and is discussed herein.

Recently, the Phase 3 report for the OECD validation of the *in vitro* steroidogenesis assay with the H295R cell line was published (Hecker et al. 2011). In this final phase of the validation, glyphosate was tested and shown not to impact steroidogenesis (i.e., estrogen or testosterone levels) in two independent laboratories. Glyphosate was also evaluated in the OECD validated Hershberger and Uterotrophic assays. The Hershberger assay screens for androgen agonists, androgen antagonists and 5α-reductase inhibitors. No evidence of androgenicity, anti-androgenicity or 5α-reductase inhibition was noted in any glyphosate treated group. The Uterotrophic assay evaluates estrogenicity by evaluating uterine weight. Uterine weights in the glyphosate treated groups were not different to the vehicle control groups. The results from both of these assays are consistent with the results of the existing two-generation rat reproduction studies.

The endocrine-modulating potential of glyphosate has been evaluated in a variety of studies, including *in vitro* assays and standard *in vivo* toxicology studies. Glyphosate has demonstrated no estrogenic, anti-estrogenic, androgenic or anti-androgenic potential in any *in vitro* assays. A review of these studies is contained herein an in the very recent paper published by Williams et al. (2012). There were also no definitive findings in the glyphosate subchronic, chronic, developmental, or reproductive toxicity studies of any endocrine-modulating effects (see Tables 3 and 4 from Williams et al. 2000; rat two gen studies summarized in section IIA part 5).

Histopathological observations of endocrine and reproductive tissues from animals in chronic and multigeneration toxicity studies indicate that glyphosate exposure had no adverse histological consequence on any reproductive or endocrine tissue from either male or female rats even at exaggerated dosage levels. Negative results were also obtained in a dominant lethal study conducted at very high doses of glyphosate. While this latter test is typically used to assess genetic toxicity, substances that affect male reproductive function through endocrine modulating mechanisms can also produce effects in this type of study (reproductive indices include mating, fertility, conception). Additionally, no effects indicative of endocrine activity were detected in a fish full life-cycle study and several avian one-generation reproduction studies performed at exaggerated dosage levels.

Several in-depth reviews on the safety of glyphosate have been conducted by several regulatory agencies and scientific institutions worldwide, and all have concluded that there is no indication that glyphosate has potential endocrine activity or will result in adverse effects on endocrine systems in humans or other mammals. The conclusions of these reviews are summarized below.

- The U.S. EPA (1998) reviewed the subchronic and chronic mammalian studies for glyphosate and concluded that there was no evidence to suggest that glyphosate produces endocrine-modulating effects.
- In a comprehensive review of the standard mammalian toxicology studies, Williams et al., (2000) concluded that glyphosate does not have the potential to produce adverse effects on endocrine systems in humans or other mammals.

- The list of purported endocrine disruptors compiled by the Institute of Environment and Health (IEH, 2005) lists glyphosate as a substance with no evidence of potential endocrine-disrupting effects.
- Following a review of the standard mammalian and wildlife toxicology studies, it was concluded in a recent ECETOC guidance document on identifying endocrine disrupting effects that glyphosate is not an endocrine disruptor using the internationally accepted Weybridge definition[89] for endocrine disruption (ECETOC, 2009).
- In a very recent of the available literature, Williams et al., (2012) concluded that there no reliable evidence linking glyphosate exposure to adverse developmental or reproductive effects at environmentally realistic exposure concentrations.

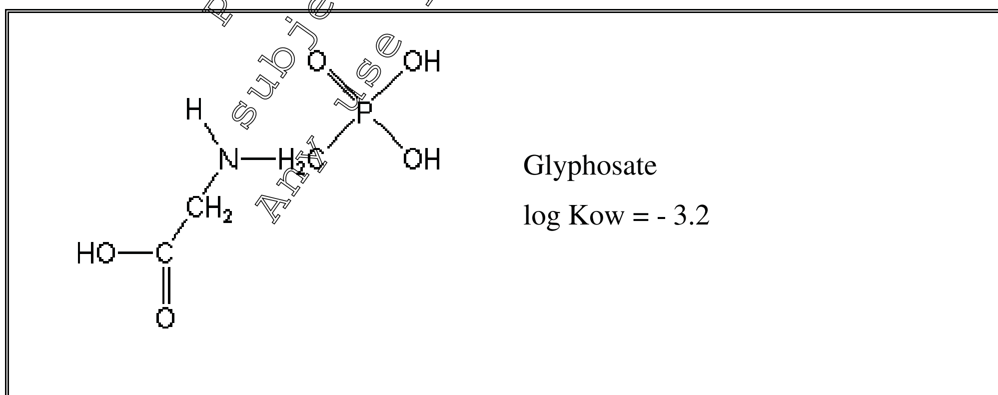
Glyphosate is not an endocrine disruptor based on structure activity relationship

OECD member countries have long recognized the potential of structure activity relationships (SARs) as an aid in conducting initial hazard assessments for chemicals, including potential endocrine activity. In August 2009, the EPA's midcontinent Division in Duluth presented a structure activity model developed to evaluate estrogen receptor binding potential. This model is an hypothesis-driven approach based on what is described as 'Expert Rules' to assign estrogen receptor binding potential and how the system provides information supporting the assignment. EPA's FIFRA Scientific Advisory Panel, with the help of the Food Quality Protection Act Science Review Board, endorsed the model as a tool to predict whether a chemical can bind to the estrogen receptor and eventually to help prioritize chemicals for Tier 1 screening under the USEPA's Endocrine Disruptor Screening Program (EDSP).

This model demonstrates that a chemical will have low potential for interaction with the estrogen receptor if,

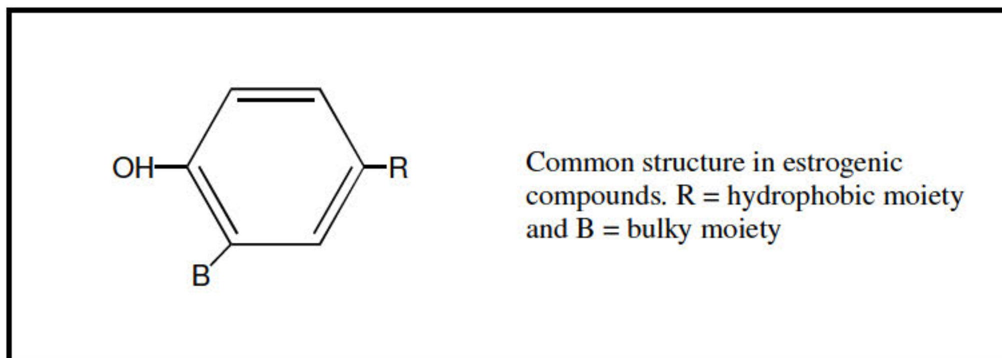
1. the chemical belongs to a group where testing showed no evidence of estrogen receptor interaction (relative binding affinity less than 0.00001%), as with acyclic molecules or cyclic molecules that do not contain a likely H-bonding group and,
2. the log K_{ow} of the chemical is less than1.3.

Molecules that have been shown to have estrogen receptor binding activity are cyclic or aromatic, having an overall ring structure as described by Blair et al., 2000; Nishihara et al., 2000; Kojima et al, 2004. Glyphosate is an acyclic molecule, as illustrated below, and, therefore, does not contain the core structure of compounds predicted to have estrogenic activity. Glyphosate also has a log K_{ow} of much less than 1.3 (log K_{ow} = -3.2). Applying the expert rules of the model to glyphosate, a relative binding affinity (RBA) of <0.00001% can be assigned, indicating essentially no potential to interact with the C. This conclusion that glyphosate does not have estrogenic potential has been empirically confirmed in a number of *in vitro* assays and *in vivo* studies.



Glyphosate
log Kow = - 3.2

[89] Weybridge (1996) "An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine function. A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."



Common structure in estrogenic compounds. R = hydrophobic moiety and B = bulky moiety

Although SAR models are not developed to the same degree for androgens as they are for estrogens, many of the same principals that apply to assessing estrogenic potential apply to assessing androgenic and anti-androgenic potential. Based on its chemical structure, glyphosate is predicted to not have androgenic activity (Devillers et al., 2009; Panaye et al., 2008). This conclusion has been empirically confirmed in a number of *in vitro* assays and *in vivo* tests. A detailed review of this data is provided in the presentation of the OSRI for each of the assays that evaluate the potential for estrogenic and androgenic activity.

The repeat dose *in vivo* toxicology studies required by EU regulatory agencies worldwide detect modulation of endocrine system activity (Carney *et al.*, 1997; Stevens *et al.*, 1997, 1998; Harvey and Johnson, 2002). These repeat dose GLP studies evaluate subacute, subchronic or chronic duration and the key endpoints of carcinogenicity, reproduction and fertility and prenatal developmental toxicity studies and provide the most robust data for toxicological evaluations. These *in vivo* studies are more predictive than *in vitro* screening assays as they assess a variety of endocrine-sensitive endpoints in animals that are capable of metabolic activation and/or detoxification. These studies also use extended exposure periods encompassing various stages of endocrine development. Endocrine-active substances affecting a single or multiple endocrine target sites invariably initiate direct or compensatory biochemical, cellular, and/or histopathological processes that will be detected in standard toxicology studies required for pesticide registration in Europe. A comprehensive histopathological assessment of endocrine tissues combined with gross organ pathology and organ weight data allows detection of all adverse endocrinopathies.

The standard toxicology and ecotoxicology studies that provide valuable information on potential endocrine-modulating effects include subchronic, chronic, and developmental and reproduction studies (Table 1). The multigeneration rat reproduction study is the most comprehensive and definitive study for evaluating the potential of substances to produce endocrine modulating effects in humans and other mammals. This study evaluates effects on gonadal development and function, estrous cycle mating behavior, fertilization, implantation, *in utero* development, parturition, lactation, and the ability of offspring to survive, develop and successfully reproduce. The comprehensive histopathological assessment of all major organ systems is a prominent feature of these studies. Developmental toxicity studies evaluate effects on many of these same processes, while subchronic and chronic studies incorporate numerous direct and indirect evaluations of endocrine and reproductive tissues, such as target organ weights and a comprehensive assessment of endocrine organ pathology.

The rat multigeneration study is the most comprehensive of the current tests for reproductive and endocrine toxicity, providing an enormous amount of information that is of the same nature as a significant portion of the data intended to be generated in many of the Tier 1 screening assays. The revised 1998 OECD test guideline for a multiple generation reproductive and developmental toxicity assay includes many developmental benchmarks predictive of endocrine disruption potential, including:

- day of vaginal opening
- mean estrus cycle length and pattern
- a diverse set of female organ weights and histopathology (ovaries, uterus, vagina, and mammary glands)
- mating and fertility indices

- number of implantation sites
- male tissue weights and histopathology (testes, epididymis, prostate, and seminal vesicles)
- male gamete parameters (e.g. sperm number, motility and morphology)

Table 1: *In vivo* ecotoxicology guideline tests that provide endpoints capable of detecting endocrine effects and a comparison to the rat multigeneration study

Endpoint	Fish Full Life Cycle Test (OPPTS 850.1500)	Avian Reproduction Test (OECD 206/OPPTS 850.2300)	Rat Multigeneration Reproduction Test
Fertilization Success	Yes	Yes	Yes
Hatchability	Yes	Yes	NA
Birth or hatching weight	No	Yes	Yes
Morphological abnormalities	Yes	Yes	Yes
Viability	Yes	Yes	Yes
Behavior	Yes (mating)	No	Yes (mating)
Growth	Yes	Yes	Yes
Onset of maturity (or puberty) or reproduction	Yes	Yes	Yes
Fecundity of offspring	No	No	Yes
Sex ratio of offspring	No	No	Yes
Intersex in offspring	No	No	Yes
Egg shell thinning	NA	Yes	NA

One of the most critical issues faced in endocrine testing is setting the appropriate dose levels or concentrations at which to conduct the screening assays. Appropriate dose setting is critical to not confuse systemic toxicity with genuine endocrine mediated effects. Therefore, dose setting takes on considerably greater importance in the testing for adverse effects on the endocrine systems than in traditional toxicity testing. The purpose of screening is to identify the potential to interact with the endocrine system via an estrogenic, androgenic or thyrogenic mode of action (and impacts on steroidogenesis), not to merely identify adverse effects. Thus, it is critical that doses are set in the range where effects on the measured endpoints are not indirectly impacted by general mechanisms of toxicity, such as liver toxicity or body weight changes, or produce an indirect effect as the result of overt toxicity or a general stress response. Any compound tested at a high enough level will impact the endocrine system; however, that impact may not be the most sensitive endpoint.

***In vitro* assays demonstrate glyphosate does not impact the estrogen receptor, androgen receptor and steroidogenesis**

Recently, several in vitro endocrine activity screening assays were performed to meet the USEPA EDSP data requirements. The results from these studies will be available (estrogen and androgen receptor binding, estrogen receptor transcriptional activation and aromatase activity. As previously mentioned glyphosate was tested in the final phase of the OECD validation of the steroidogenesis assay and shown not to impact steroidogenesis. However, functionally equivalent data to that which can be obtained by performing the estrogen receptor binding assay and the estrogen receptor transcriptional activation assay are available for glyphosate in three independent studies reported in peer-reviewed journals. These studies clearly demonstrate that glyphosate does not possess estrogenic or anti-estrogenic potential. The estrogenic and anti-estrogenic potential of glyphosate has been studied *in vitro* by independent researchers and published in peer-reviewed journals. The methodology used in these studies (Kojima et al. 2004; Petit et al. 1997) is functionally equivalent to the estrogen receptor binding and transactivation assays defined in the Tier 1 battery of the EDSP. Even though the in vitro data was generated with different experimental methods it is of suitable nature and quality to provide the same essential predictive to evaluate potential estrogenic and anti-estrogenic potential for glyphosate. The results from both of these investigations

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provides strong evidence that glyphosate is not an estrogen receptor α/β agonist or antagonist. A comprehensive review of these assays follows.
IC_{50} value for estradiol in estrogen receptor competitive binding assays and transactivation assays are comparable to the IC_{50} value for the OPPTS 890.1250 estrogen receptor competitive binding assay and the OPPTS 890.1300 transcriptional activation assays, respectively. The measurement endpoints for these two assays are highly correlated and demonstrate similar sensitivity and specificity (Sonneveld et al., 2006; Takeyoshi, 2006; Laws et al., 2006). This result is plausible and not unexpected since both assays are measuring the same functional endpoint, receptor-ligand binding.

ER binding and transactivation assays have been shown to be good predictors of *in vivo* estrogenic activity (Sonneveld et al., 2006; Takeyoshi, 2006). This is particularly true for compounds where metabolism studies in rodents have shown that they are poorly metabolized[90], are not substantially absorbed when ingested, are rapidly excreted, and do not accumulate in the body (EPA, 1993; William et al., 2000, Ridley and Mirly, 1988), as is the case for glyphosate.

Kojima, H., Katsura, E., Takeuchi, S., Niiyami, K., and Kobayashi, K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environ. Health Perspect. 112:524-531.

The ability of glyphosate to act as agonist and antagonist of the human estrogen receptor α and human estrogen receptor β isoforms was investigated by Kojima et al. using Chinese hamster ovary (CHO-K1) cells that were transiently transfected with an estrogen responsive reporter vector. The estrogen responsive element containing reporter plasmid was constructed as described by Kojima et al., (2003). For detection of estrogen receptor α and estrogen receptor β activity, cells were transfected with 5 ng of estrogen receptor α or estrogen receptor β expression vectors and 50 ng of the estrogen receptor reporter construct. Additionally, a pRL-SV40 construct containing the *Renilla* luciferase gene was co-transfected to evaluate transfection efficiency and to discriminate between specific down regulation from cytotoxicity.

Three hours post-transfection, cells were dosed for 24 hours with concentrations of glyphosate ranging from 10^{-5} to 10^{-8} M (i.e., 0.01 µM to 10 µM). The 24-hour exposure duration for this assay is the same as that required by the OPPTS 890.1300 estrogen receptor transactivation guideline. Transfected cells were cultured in complete media, containing penicillin-streptomycin and 10% charcoal-dextran-treated fetal bovine serum at 37°C in an atmosphere of 5% CO_2/95% air under saturated humidity. The approximate EC_{50} values for estrogen receptor α and estrogen receptor β were 1 and 10 pM, respectively, which are 6 to 7 orders of magnitude greater than the highest glyphosate concentration tested. Parallel assays were performed to fully characterize the concentration effect relationship with estradiol for estrogen receptor α and estrogen receptor β (Figure 1).

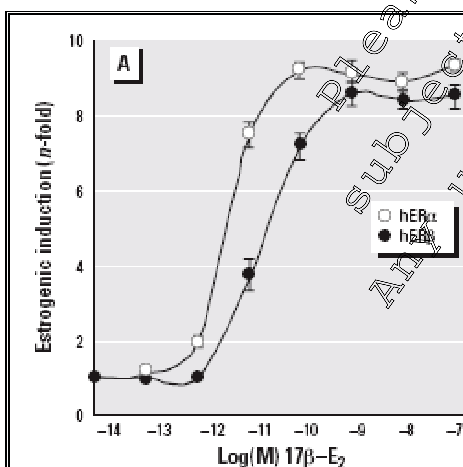

Figure 1. Concentration–response curves for 17β-estradiol in the estrogen receptor α and estrogen receptor β assays in Chinese hamster ovary cells transiently transfected with human estrogen receptor α or human estrogen receptor β, the estrogen receptor luciferase reporter vector and a constitutively active *Renilla* luciferase expression reporter (transfection and toxicity control). Values represent the mean ± SD of three independent experiments and are presented as mean n-fold induction over the vehicle control.

The sensitivity of this assay and dynamic range are comparable to the sensitivity and dynamic range of other estrogen receptor reporter assays reported in the open literature (Legler et al., 1999; Rogers et al., 2000; Wilson et al., 2004; Gordon and Clark, 2005) and to that of the OPPTS 890.1300 estrogen receptor transactivation guideline (Takeyoshi, 2006). Also, the IC_{50} value reported by Kojima et al. for the transactivation assay for glyphosate is comparable to the IC_{50} value reported by Laws et al. using the OPPTS 890.1250 estrogen receptor competitive binding assay (Laws et al., 2006). This is plausible and not unexpected since both assays are measuring the same functional endpoint, receptor-ligand binding.

Agonist activity of glyphosate was evaluated by relative activity expressed as the 20% relative stimulatory concentration (EC_{20}); that is, the concentration of glyphosate showing 20% of the activity of maximally inducing concentrations of 10^{-10} M estradiol for human estrogen receptor α and 10^{-9} M estradiol for estrogen receptor β activity, respectively. Based on this criterion, glyphosate was not identified as a chemical having estrogen receptor α and estrogen receptor β antagonist activity (Kojima et al, 2004).

Petit F., LeGoff P., Cravedi J.P., Valotaire Y., F. Pakdel. (1997). Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J. Molecular Endocrinology. 19: 321-335.

Petit et al. (1997) screened glyphosate for estrogenic potency using two *in vitro* systems: a recombinant yeast system expressing the rainbow trout estrogen receptor. Yeast cells containing a *lacZ* reporter gene linked to two estrogen-responsive elements were treated in culture at 10^{-8} to 10^{-4} M (0.01 to 100 µM) glyphosate for four hours. For these assays, 17β-estradiol was used as the positive control and produced good responsiveness with approximately a 100-fold induction of β-galactosidase activity. To ensure that the absence of a response was not due to toxicity, cell density measurements were made before and after treatment. The estradiol concentration required to produce maximal activation was 10^{-8} M and was reported as 100% activity. Basal induction represented 14 to 18% of the maximum activity obtained with the receptor in the absence of ligand**.** The reported activity for glyphosate on estrogen dependent gene transactivation was reported to be 15.84%, well within the range of basal activity, indicating no evidence of estrogenic activity.

Xie L, Thrippleton K, Irwin MA, Siemering GS, Mekebri A, Crane D, Berry K, Schlenk D. (2005). Evaluation of estrogenic activities of aquatic herbicides and surfactants using an rainbow trout vitellogenin assay. Toxicol Sci. 87:91-8.

In Xie et al. (2005), the estrogenic potency of glyphosate was evaluated using vitellogenin as a biomarker of exposure for juvenile rainbow trout (standard length: 11.5 ± 2.2 cm) exposed to a mean measured concentration of 0.11 mg/L glyphosate. The analytical method used for measuring the concentration of glyphosate in water was EPA Method 547 with a limit of detection of 0.0050 µg/L and recovery ranging from 80-87%.

Rainbow trout were exposed for 7 days at 16 ± 1°C in 20-L tanks under static-renewal and received water exchanges daily. In addition, rainbow trout were exposed to five concentrations of 17β-estradiol to characterize the concentration-effect relationship for 17β-estradiol. Each concentration of chemical (treatment or control) had three replicates with two fish in each tank. The test water in each individual tank was monitored daily for water chemistry after every water renewal. The hardness of the test water ranged from 142 to 162 mg/l (as CaCO3); the free chlorine was <0.2 mg/l. Alkalinity ranged from 148 to 180 mg/l, and ammonia (as N-NH3) was <0.02 mg/l. Dissolved oxygen averaged 94.6% of the air saturation value, and pH values ranged from 6.0 to 8.2 mg/l. Fish were fed trout chow equivalent to approximately 1% of their body weight during the exposure and held under a 16 h:8 h (light:dark) photoperiod. The results of this study demonstrate that glyphosate did not induce production of vitellogenin, providing additional evidence that glyphosate does not have estrogenic activity (Figure 2).

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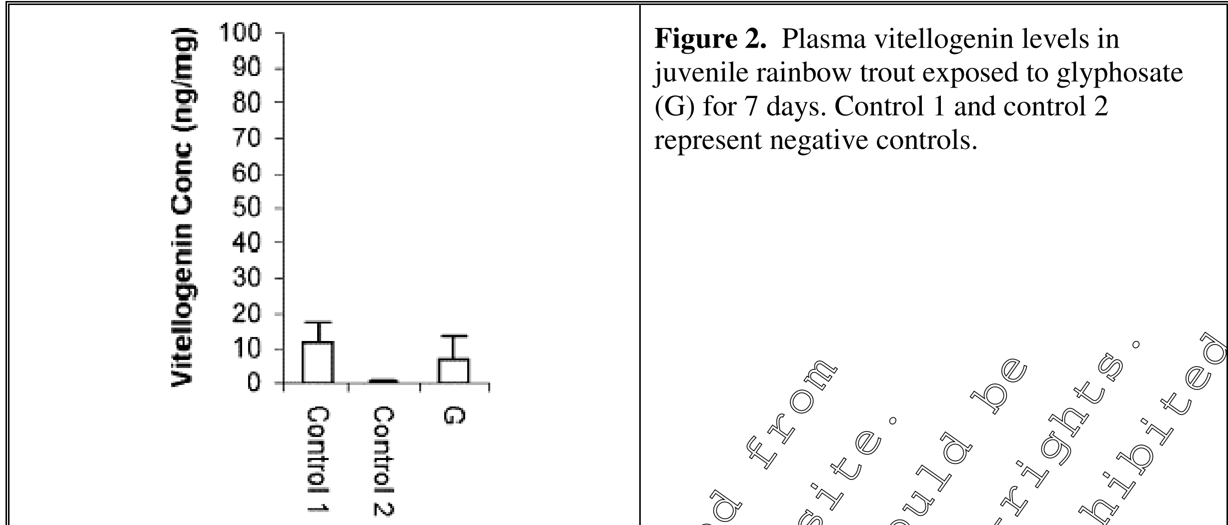

Figure 2. Plasma vitellogenin levels in juvenile rainbow trout exposed to glyphosate (G) for 7 days. Control 1 and control 2 represent negative controls.

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An independent study that is functionally equivalent to the Androgen Receptor Binding Assay of the EDSP and published in Environmental Health Perspectives by Kojima et al. in 2004 clearly demonstrates the lack of any antagonistic androgen receptor activity for glyphosate.

Kojima, H., Katsura, E., Takeuchi, S., Niiyami, K., and Kobayashi, K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese hamster ovary cells. *Environ. Health Perspect.* 112:524-531.

The ability of glyphosate to act as agonist and antagonist of the human androgen receptor was investigated using Chinese hamster ovary (CHO-K1) cells that were transiently transfected with an androgen responsive reporter vector (Kojima et al., 2004). The androgen responsive element containing reporter plasmid was constructed as described in Kojima et al., (2003). To evaluate agonist and antagonist effects of androgen receptor activity, cells were transfected with 2.5 ng of androgen receptor expression vectors and 50 ng of the androgen receptor reporter construct. Additionally, a pRL-SV40 construct containing the *Renilla* luciferase gene was co-transfected to evaluate transfection efficiency and to distinguish between specific down regulation from cytotoxicity.

At three hours post-transfection, cells were dosed for 24 hours with concentrations of glyphosate ranging from 10^{-5} to 10^{-8} M. This 24-hour exposure is the same as that required by the androgen receptor transactivation guideline OPPTS 890.1150. Transfected cells were cultured in complete media containing penicillin-streptomycin and 10% charcoal-dextran-treated fetal bovine serum at 37°C in an atmosphere of 5% CO2/95% air under saturated humidity.

The approximate IC_{50} value for the androgen receptor was approximately 6 orders of magnitude greater than the highest glyphosate concentration tested. Parallel assays were performed to fully characterize the concentration effect relationship with estradiol for androgen receptor (Figure 3).

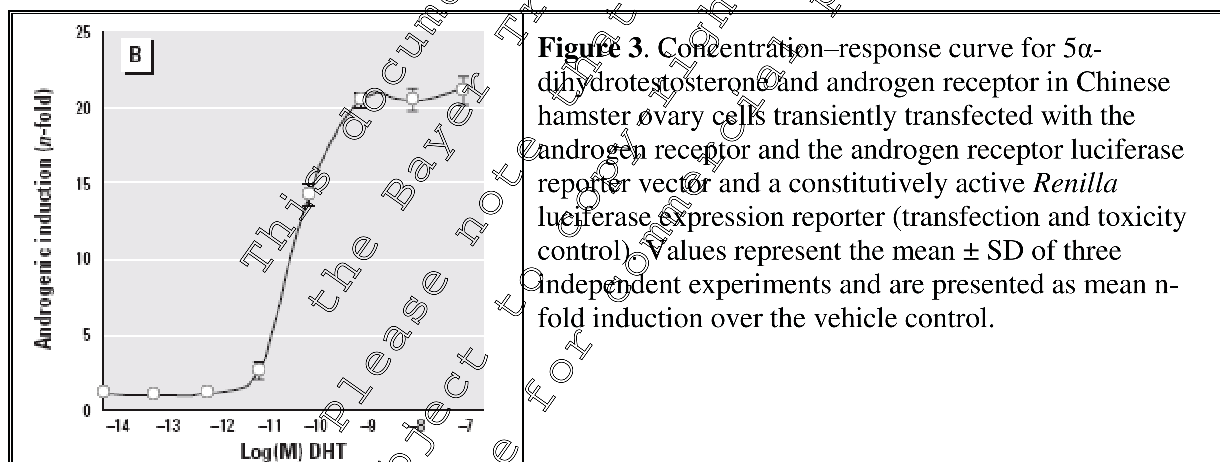


Figure 3. Concentration–response curve for 5α-dihydrotestosterone and androgen receptor in Chinese hamster ovary cells transiently transfected with the androgen receptor and the androgen receptor luciferase reporter vector and a constitutively active *Renilla* luciferase expression reporter (transfection and toxicity control). Values represent the mean ± SD of three independent experiments and are presented as mean n-fold induction over the vehicle control.

The sensitivity of this assay and dynamic range is comparable to the sensitivity and dynamic range of other standard androgen receptor reporter assays in the open literature (Beck et al., 2008). Additionally, the IC_{50} value for the transactivation assay conducted by Kojima et al. is comparable to the IC_{50} value for the AR competitive binding assay conducted according to OPPTS guideline 890.1150 (Araki et al., 2005; Sonneveld et al., 2005; Beck et al., 2008). This is plausible and not unexpected since both assays are measuring the same functional endpoint, receptor-ligand binding.

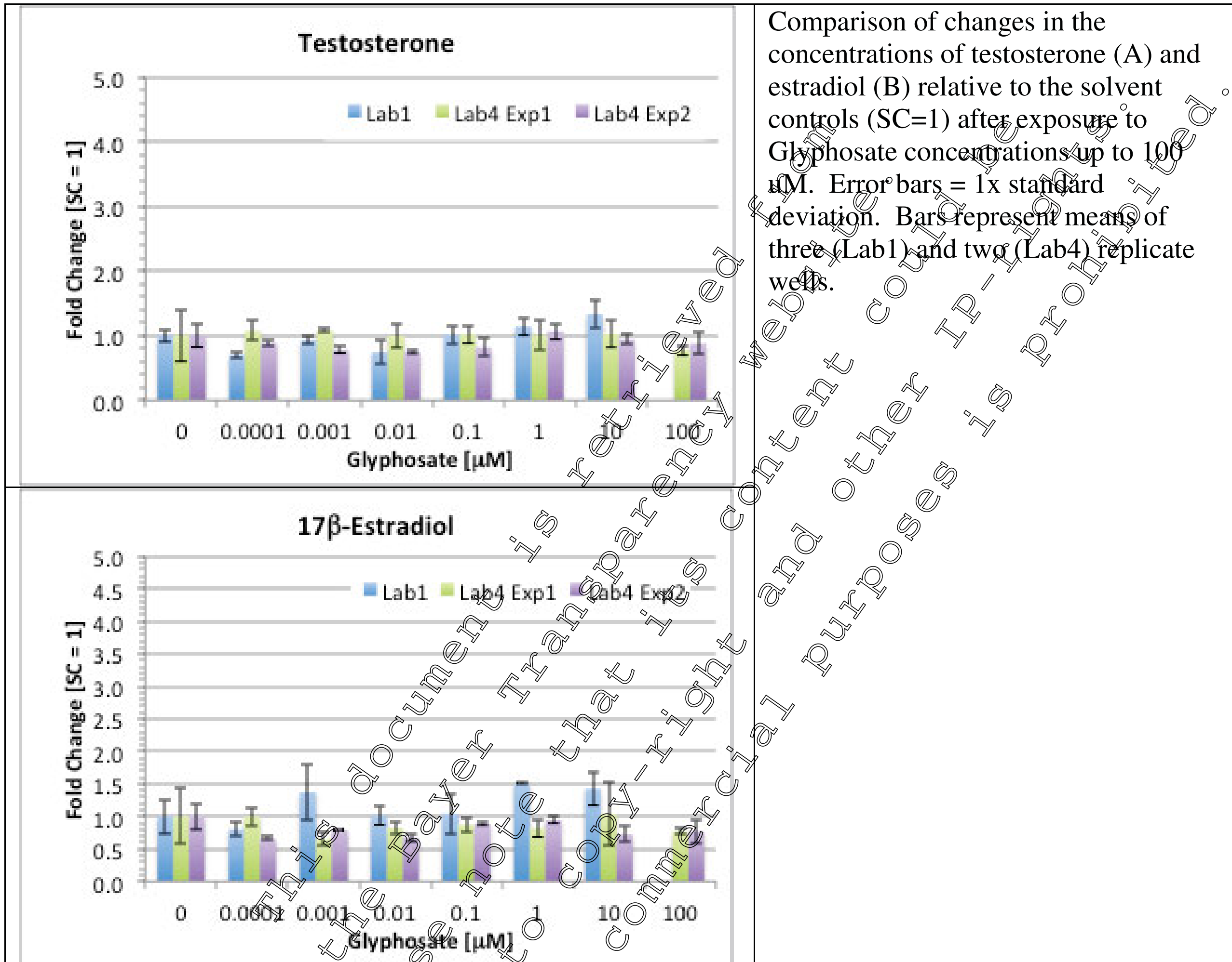
Agonist activity was evaluated by relative activity expressed as the 20% relative stimulatory concentration (IC_{20}); that is, the concentration of glyphosate showing 20% of the activity of maximally inducing concentrations of 10^{-10} M dihydrotestosterone**.** Based on this criterion, glyphosate was not identified as a chemical having androgen receptor antagonist activity (Kojima et al, 2004).

Conclusion – Estrogen Receptor Agonism and Antagonism
Data generated by Kojima et al. (2004), Petit et al. (1997) and Xie (2005) in peer-reviewed

journals clearly and consistently demonstrate that glyphosate does not have estrogen and androgen receptor binding potential, either as an agonist or antagonist.

Steroidogenesis

As previously discussed glyphosate was shown not to impact steroidogenesis in the phase 3 validation of OECD's H295R assay (Hecker at al., 2011). A review of the result presented in the publication is illustrated below.



Comparison of changes in the concentrations of testosterone (A) and estradiol (B) relative to the solvent controls (SC=1) after exposure to Glyphosate concentrations up to 100 µM. Error bars = 1x standard deviation. Bars represent means of three (Lab1) and two (Lab4) replicate wells.

The published study Richards' et al. 2005 is not indicative of inhibition of aromatase activity

A research published by Richard et al. (2005) purported inhibition of aromatase (CYP19) activity by glyphosate. However, this data is not indicative of inhibition of aromatase activity. The test concentrations used by Richard et al. were solutions of 0.01, 0.05, 0.1, 0.5, 1 and 2% glyphosate by weight, which correspond to glyphosate molar concentrations of 213, 1064, 2129, 10645, 21289, 42578 µM, respectively.[91] No inhibition of aromatase activity was detected at glyphosate concentrations of 213, 1064, 2129 µM. <u>At the higher tested concentrations the effects on aromatase activity were very likely driven by a pH effect. In other words, these very high concentrations of glyphosate acid dramatically lowered the pH of the assay buffer and likely result in denaturation of the aromatase enzyme and associated reductases that provide reducing equivalents for the substrate transformation. Method development work done in preparation of the aromatase assay performed for the EDSP confirm this conclusion.</u> Additionally, the highest concentration required to be tested by the EDAP is 1000 µM, an extremely high test concentration. Therefore, it is not appropriate to conclude based on the results of Richard et al. (2005) that glyphosate inhibits *in vitro* aromatase activity. The conclusion of no impact on aromatase activity is also supported by the recent steroidogenesis work of Hecker at al (2005), that evaluated aromatase activity in cell culture, and the results of the *in vivo* studies discussed below.

[91] Example calculation for 1% treatment: 0.01 * 360 g a.e./L * mole/169.1 * 10^6 µmoles/mole = 21,289 µM

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In vivo studies demonstrate the glyphosate does not impact reproduction and development at exposure concentrations that greatly exceed environmentally realistic field concentrations

Mammalian multigeneration studies

Considerable value is found in multigenerational reproduction studies for the evaluation of the potential for a substance to potentially interact with the endocrine system. Many such studies have been conducted to evaluate glyphosate and are reviewed in section IIA part 5. Multigenerational studies cover a range of specific and apical endpoints, which may be used to evaluate endocrine activity, including behavioral changes, signs of difficult or prolonged parturition, ability to become pregnant, duration of gestation, sex ratio of the offspring, feminization or masculinization of offspring, number of pups, stillbirths, and gross pathology and histopathology of the vagina, uterus, ovaries, testes, epididymis, seminal vesicles, prostate, and any identified target organs (Stevens et al., 1997). The rat multigenerational study is the most comprehensive of the current tests for reproductive and endocrine toxicity, providing an enormous amount of information that is of the same nature as a significant portion of the data intended to be generated in many of the Tier 1 screening assays. The revised 1998 U.S. EPA guideline (OPPTS 870.3800) for a multiple generation reproductive and developmental toxicity assay includes many developmental benchmarks predictive of endocrine disruption potential. These multigenerational studies comprehensively assess endocrine functions that are required for reproduction, development and chronic health and provide no evidence of endocrine activity. One of the key recent multigenerational studies was by Moxon (2000). Moxon (2000) conducted a multigenerational reproduction study in Wistar derived rats (26 rats/sex/group), which is compliant with the current 1998 US EPA OPPTS 870.3800 guideline. Dietary exposure to glyphosate at 0, 1000, 3000 and 10000 parts per million (ppm) had no adverse effect on reproductive performance, including:

- day of age when vaginal opening occurred in the F1 females
- unadjusted weight and organ to body weight ratio of the ovaries or uterus (including cervix) of the F0 and F1 parent animals
- number of primordial and small growing follicles of the left ovary of the F1 parent females
- macroscopic or microscopic findings observed in any tissue from the F0 or F1 animals, including uterus, cervix, ovaries and vagina
- matings in all treatment groups
- ratio of male/female pups born
- litter size during lactation

Conclusion

There is consistent evidence showing a lack of estrogenic, androgenic, thyrogenic and steroidogenic activity of glyphosate in these multigenerational studies in rats.

Fish full Life cycle study

A fish full life cycle study has been performed for glyphosate. The fish full life-cycle study is the highest Tier of fish ecotoxicology testing and typically performed with the fathead minnow (Pimphales promelas) as a freshwater test species. In the full life-cycle study, the entire life-cycle is exposed; therefore, de facto the most sensitive life-stage to any toxicant (including endocrine active compounds) is encompassed by the test design.

In the first or parental generation (F_0) the following endpoints are evaluated: embryo hatching success, embryo time-to-hatch, survival at various time points after hatch, length and wet weight as an indicator of growth at various time points after hatch, and reproductive success (fecundity) as measured by number of eggs per female per day, number of spawns and number of eggs per spawn. In the second generation (F_1)

the endpoints include embryo hatching success, embryo time-to-hatch, survival, and development as measured by length and wet weight at specified time points.

Fathead minnows (F_0 + F_1) were continuously exposed to a mean measured glyphosate concentrations of 0.7, 2.8, 7.0, 13.0 and 25.7 mg/L for 255 days. The current guidance in OPPTS 890.1350 states ‘to test the highest concentration that does not result in increased mortality or signs of overt morbidity compared to the control, or 1/3 the derived 96-hr LC_{50} will serve as the highest exposure concentration in the 21-day test.” In a static 24-hour acute study run in advance of the full life-cycle study, using ~1.5 g fathead minnows, the 24-hour LC_{50} of glyphosate was determined to be 84.4 mg/L and mortality was shown to result from the low pH at the highest test concentrations. Therefore, the highest tested concentration in the full life-cycle study was approximately 1/3 the acute LC_{50} value. This conclusion is supported by the range of acute toxicity values for other fish species reported in the Glyphosate Monograph and section IIA 8.2. Additionally, glyphosate has been shown to not bioconcentrate in fish, with a bioconcentration factor in whole fish of less than 1X.

The full life-cycle study with fathead minnows exposed to glyphosate began with two groups of thirty fertilized eggs incubated in each test aquarium at 25°C. Post hatch, fish were randomly divided into two groups of twenty and distributed to growth chambers until sexual maturity was reached. When secondary sexual characteristics were well developed (*circa* day 134) the number of fish in each tank was reduced initially to 4 males and 4 females, and subsequently to 2 males and 4 females. This ratio of 2 males to 4 females is required by the OPPTS 890.1350 guideline.

When spawning began, eggs were removed from the underside of spawning tiles daily and eggs of each spawn were counted. Fifty eggs from each of the first ten spawning in each tank were then oscillated in their respective test waters by means of the egg cup and rocker arm apparatus until hatching was completed (3-5 days at 25°C). After 30 days exposure, fry groups were terminated and total lengths determined by the photographic method. Total wet weight and percent survival were also determined at this time for each fry group. Total length, wet weight, sex and gonadal conditions were determined for each adult fish at the termination of the experiment.

Continuous exposure to concentrations of glyphosate as high as 25.7 mg/L had no statistically significant effects on any of the parameters studied during 254 days of continuous exposure. Hatchability of eggs was excellent (>94%) in all concentrations of glyphosate and controls. Per- cent survival and total length of fathead minnows after 30 and 60 days exposure to concentrations of glyphosate as high as 25.7 mg/L did not differ significantly from the control .

Survival of the fifteen original fathead minnows placed in each spawning chamber was excellent in all concentrations of glyphosate and controls through day 134, when fish were thinned to a ratio of 4 males and 4 females. At termination, total length and wet weight of the female fathead minnows exposed to all concentrations of glyphosate were similar to controls.

Spawning began in many tanks around day 112 and had occurred in all tanks by day 148 of exposure. The number of spawnings, eggs per female and eggs per spawn did not differ significantly between controls and fish exposed to concentrations of glyphosate as high as 25.7 mg/L. One spawn of 33 eggs was recovered from the B replicate of 25.7 mg/L before the accidental death of fish due to a diluter malfunction early in the spawning period. Prior to that time, all fish appeared healthy and had reached sexual maturity. Percentage of live fry hatching in concentrations of glyphosate as high as 25.7 mg/L was similar to that which was observed in the controls. Although gonadal histopathology was not evaluated in the fish full life cycle study, absence of effects on the number of spawns, eggs per female and eggs per spawn provides strong evidence of no impact on gonadal structure and function.

Survival and total length and wet weight of second generation (F_1) fathead minnows was similar to controls for fish exposed 30 days to concentrations of glyphosate as high as 25.7 mg/L. Based on the absence of any significant effect to fathead minnows chronically exposed to glyphosate, the no observed effect concentration (NOEC) for this species was determined to be >25.7 mg/L, the highest concentration tested.

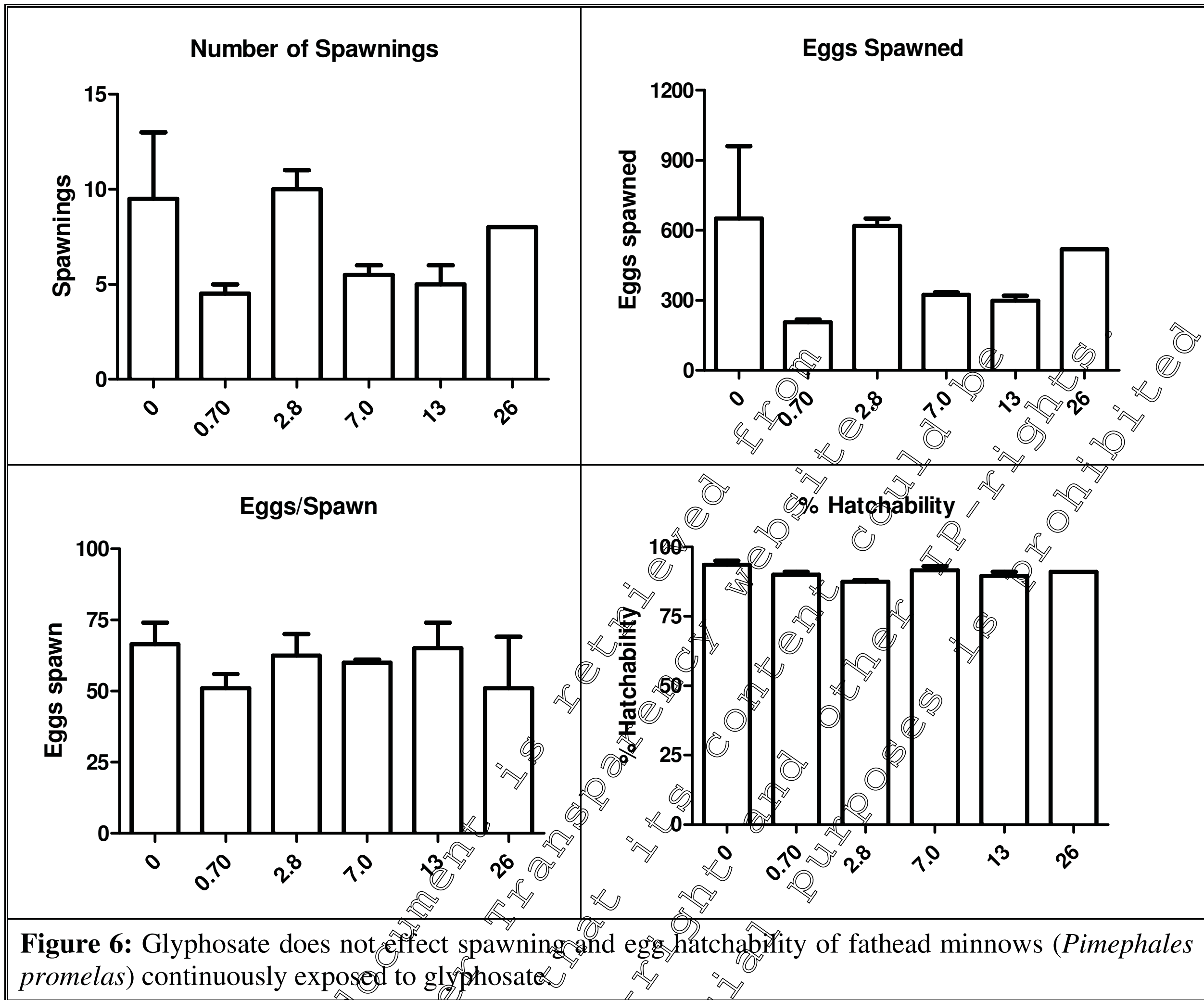

Figure 6: Glyphosate does not effect spawning and egg hatchability of fathead minnows (*Pimephales promelas*) continuously exposed to glyphosate.

Summary – Fish Full Lifecycle Study

Fathead minnows (F_0 + F_1) continuously exposed to high concentration of glyphosate for 255 days demonstrated no treatment-related effects on the survival, growth and egg production of first generation fish or on hatchability, survival and growth of second-generation eggs and fry. Observing no effects on any of these endpoints over this concentration range, provides strong evidence that glyphosate does not have estrogenic, anti-estrogenic, androgenic or anti-androgenic activity. Further, as discussed in detail in an earlier section, the structure activity relationship of glyphosate predicts no estrogenic activity nor is glyphosate able to transactivate an estrogen receptor responsive report gene. Additionally, an absence of any detectable effects on growth, development and reproduction demonstrates a lack of an effect on effect on steroidogenesis, gonadal function and the hypothalamic-pituitary-gonadal (HPG) axis function.

Additional relevant information that supports the findings of the fish full-life cycle study is reviewed in the following sections. This includes information from *in vitro* and *in vivo* studies examining the potential for estrogenicity and a review of the avian one-generation studies.

Structure activity relationship indicates lack of estrogenicity

As previously discussed in the Introduction, glyphosate does not have the structure of a molecule predicted to have estrogenic or androgenic activity. Although SAR models are not developed to the same degree for androgens, many of the same principals that apply to evaluating glyphosate for estrogenic potential apply to an assessment of androgenic potential. Based on the structure of glyphosate is predicted not to have androgenic activity (Devillers et al., 2009; Panaye et al., 2008). The conclusion that glyphosate does not have estrogenic and androgenic potential has been empirically confirmed in a number

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of *in vitro* assays and *in vivo* tests. A full review of the *in vitro* data and *in vivo* data is provided in the presentation of the OSRI for each of the assays that evaluate the potential for estrogenic activity.

Glyphosate does to induce production of vitellogenin

The estrogenic potency of glyphosate was evaluated *in vivo* with rainbow trout by measuring vitellogenin levels post glyphosate exposure. As previously discussed seven days of exposure to 0.11 mg/L glyphosate had no effect on vitellogenin levels, indicating that glyphosate is not estrogenic (Xie et al, 2005). Consistent with this result, Petit et al. (1997) demonstrated that glyphosate does not bind to and transactivate a rainbow trout estrogen receptor.

Additional supporting information of glyphosate to evaluate the potential for endocrine activity, particularly the fish short term reproduction study, is supported by four separate one-generation avian reproduction studies that followed OECD 206 guidelines. In these studies, birds were fed three diet concentrations containing the glyphosate throughout a chronic exposure period. Birds are induced, by photoperiod manipulation, to lay eggs and the eggs are collected over a ten-week period. Eggs are incubated and hatched, and the young maintained for 14 days. Mortality of adults, egg production, cracked eggs, egg shell thickness, viability, hatchability and effects on young birds are observed during the study. The endpoints that are assessed and evaluated in the avian reproduction study and the fish full life-cycle study are summarized in the table below. From this comparison it is clear that the avian reproduction study provides significant and relevant information to evaluate the potential for reproductive effects in birds. Reading across to the mammalian multigenration study provides an additional list of endpoints capable of detecting endocrine activity that are evaluated.

The original reproduction studies with northern bobwhite and mallard duck included a test concentration at the highest nominal level to test in OECD 206 of 1,000 mg glyphosate/kg feed. Two newer reproduction studies with northern bobwhite and mallard duck were conducted with the highest tested dietary concentration of 2,250 mg glyphosate/kg feed. In all four of these studies, no effects were observed on measured endpoints (that included the numbers of eggs laid, eggs damaged/laid, eggs set, viable embryos, live 3 week embryos, hatchlings, 14-day survivors, eggs laid/female, eggs laid/female/day, 14-day survivors/female and egg shell thickness. The results from these avian reproduction studies are consistent with the results from the fish full-life cycle study and further provide no evidence of potential endocrine activity even at extremely high concentrations and in long-term continuous feeding studies.

Overall Summary and Conclusions

Based on this weight-of-evidence analysis from glyphosate's extensive toxicological database, there is sufficient exiting relevant that is functionally equivalent and of the same nature and quality as that developed in an endocrine screening battery to conclude that glyphosate does not have endocrine-modulating potential. The endocrine-modulating potential of glyphosate has been evaluated in a variety of studies including *in vitro* assays and standard in vivo toxicology studies that are functionally equivalent to the data generated in the Tier 1 screening battery. The *in vivo* studies comprehensively assess endocrine functions that are required for reproduction, development and chronic health. Glyphosate has demonstrated no estrogenic, anti-estrogenic, androgenic and anti-androgenic potential in *in vitro* assays, and there was no indication of changes in endocrine function or endocrine organ histopathology in any of the *in vivo* studies. Additionally, structure activity models predict that glyphosate does not have estrogenic or androgenic activity.

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Developmental and Reproductive Toxicity (DART) and Endocrine Disruption (ED) Publications

Publications suggesting glyphosate or glyphosate based formulations are developmental toxicants, reproductive toxicants or endocrine disruptors include *in vitro* studies, *in vivo* studies and epidemiological studies with weak, statistically non-significant associations. Many of these published since 2000 are specifically discussed in a comprehensive glyphosate DART review publication by three internationally recognized experts (Williams et al., 2012), referenced in Doc L Table 3 and included in Doc K. Further discussions of some significant papers follow.

In addition, glyphosate was included on the US EPA Endocrine Disruptor Screening Program's (EDSP) first list of 67 compounds to Tier 1 Screening. The US EPA clearly published the criteria for inclusion on List 1 was strictly based on exposure potential, not hazard, specifically stating in the Federal Register (2009);

> "*This list should not be construed as a list of known or likely endocrine disruptors*".

A consortium of glyphosate registrants in North America, the Joint Glyphosate Task Force, LLC (JGTF), coordinated the conduct of the glyphosate battery of Tier 1 screening assays under the EDSP and submitted these successfully completed assays to the US EPA. The US EPA will evaluate the full battery of Tier 1 screening assays together using a weight of evidence approach, for glyphosate's potential to interact with the estrogen, androgen and thyroid endocrine pathways. The following below were submitted by the JGTF to the US EPA in early 2012 and are expected to be reviewed this year. However, the Agency gas announced they will not release their Data Evaluation Records (DERs) for individual EDSP studies until a weight of evidence review has been completed for List 1 compounds. Therefore, in an effort to disclose the findings of the glyphosate EDSP data to the scientific community, the JGTF is considering publishing a Weight of Evidence review of glyphosate with respect to endocrine disruption.

<u>*In Vitro* EDSP Glyphosate Studies submitted to the US EPA</u>

- Androgen Receptor Binding (Rat Prostate Cytosol); OCSPP 890.1150
- Aromatase (Human Recombinant); OCSPP 890.1200
- Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC); OCSPP 890.1250
- Estrogen Receptor Transcriptional Activation (Human cell Line, HeLa-9903); OCSPP 890.1300; OECD 455
- Published OECD Validation of the Steroidogenesis Assay (Hecker et al., 2010)

<u>*In Vivo* EDSP Glyphosate Studies submitted to the US EPA</u>

- Amphibian Metamorphosis (Frog) OCSPP 890.1100; OECD 231
- *In Vivo* Hershberger Assay (Rat); OCSPP 890.1600; OECD 441
- Female Pubertal Assay; OCSPP 890.1450; OECD None
- Male Pubertal Assay; OCSPP 890.1500
- Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440
- Fish Short-Term Reproduction Assay; OCSPP 890.1350; OECD 229

The glyphosate Tier 1 screening assay study reports are owned by the JGTF. The European Glyphosate Task Force (GTF) is negotiating to procure access rights to the battery of glyphosate EDSP Tier 1 screening study reports. Results of the Hershberger and Uterotrophic *in vivo* rat studies, now in the public domain, as are the published results of the OECD validation of the Steroidogenesis assay, in which glyphosate clearly had no impact on steroidogenesis, are discussed below.

***In Vitro* Glyphosate DART/ED Publications**

Many *in vitro* research publications have characterized pesticide <u>formulations</u>, including glyphosate based formulations, as toxic and endocrine disrupting products. Researchers and editorial boards have frequently overlooked the fact that surfactants (which are often components of formulated pesticide

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products), by their physico-chemical nature as surface active substances, are not suitable test substances using *in vitro* cell models. Surfactants compromise the integrity of cellular membranes, including mitochondrial membranes, and thus confound endpoint measurements considered as representative of specific toxicological modes of action or pathways. For example, Walsh et al. (2000) published research claiming that a glyphosate based formulation, but not glyphosate alone, adversely affected the steroidogenesis pathway by inhibiting progesterone production resulting in downstream reduction in mitochondrial levels of StAR protein. Subsequent research by Levine et al. (2007) demonstrated (i) no synergism between glyphosate and the surfactant since the cytotoxic effects were completely independent of glyphosate; identical dose-response curves were noted for formulated product with and without the glyphosate active ingredient; (ii) comparable cytotoxicity dose-response curves for several common household detergents or surfactants; and (iii) a variety of surfactants demonstrate cytotoxic effects that are not specific to biochemical pathways within intact cells. Levine (2007) concludes by emphasizing the importance of considering the biological plausibility of observed *in vitro* effects for in-tact animals.

Take away message: when formulations are tested, biochemical endpoints are impacted by cyctoxic effects of the surface active component of the formulation and confound the results of the assay. A significant issue is that these investigations do not use the appropriate cytotoxicity assays or interpret the results of those assays appropriately. Different cytotoxicity assay are capable of measuring early, intermediate and late stages of cytotoxicity. Signficant cytotoxicity is a valid indication not to interpret biochemical results without caution. This concept is explained and shown in Levine et al. 2007.

Subsequent research addressing the steroidogenesis pathway confirmed glyphosate lacked endocrine disruption potential specific to this pathway. Quassinti et al. (2009) evaluated effects on gonadal steroidogenesis in frog testis and ovaries on glyphosate and another active substance, noting that glyphosate unequivocally demonstrated no effect. Forgacs et al. (2012) also tested glyphosate alone and demonstrated no effect on testosterone levels in BLTK1 murine leydig cells *in vitro*. Furthermore, the OECD multi-laboratory validation of the Steroidogenesis Assay used for Tier 1 screening of the US EPA EDSP, evaluated glyphosate and concluded no impact on steroidogenesis (Hecker et al., 2010). Consequently, the US EPA considered reference to the OECD validation report sufficient for meeting the glyphosate Steroidogenesis Assay Test Order in the EDSP Tier 1 screening of glyphosate.

The Seralini laboratory at the University of Caen, France, has multiple recent publications of *in vitro* research with glyphosate and glyphosate based formulations (Richard et al, 2005; Benachour et al, 2007; Benachour and Seralini, 2009; Gasnier et al, 2009; Gasnier et al, 2010; Gasnier et al., 2011; Clair et al., 2012; Mesnage et al., 2012), with proposed extrapolations to an array of *in vivo* effects including potent endocrine disruption, aromatase inhibition, estrogen synthesis, placental toxicity, foetotoxicity, embryotoxicity and bioaccumulation. These publications are often replicates of earlier studies, using different cell lines or primary cell cultures and in some cases the same data are reported again in a subsequent publication. Firstly, the *in vitro* synergism claims are conjecture, simply because no control groups of surfactant without glyphosate were tested. Secondly, the extrapolations to *in vivo* effects are unjustifiable based on both the unsuitability of surfactants in such test systems and the supraphysiological cytotoxic concentrations at which *in vitro* effects are reported. Again often overlooked by *in vitro* researchers and editorial boards, Levine et al. (2007) presented convincing data demonstrating a lack of *in vitro* synergism for glyphosate with other formulation ingredients. Regarding Seralini's repeated claims of glyphosate induced aromatase inhibition in mircosomes (Richard et al, 2005; Benachour et al, 2007; Gasnier et al, 2009), the data are confounded and thus uninterruptable where surfactants are introduced to such *in vitro* systems. This is noted in the US EPA Aromatase Imbibition Test Guideline, OECD 890.1200, in which notes,

> "Microsomes can be denatured by detergents [surfactants]. Therefore, it is important to ensure that all glassware and other equipment used for microsome preparations be free of detergent residue."

Research from the Seralini laboratory has repeatedly gained general public and media attention, including dissemination on web sites and public lecture tours in various countries, in which allegations against glyphosate based products and biotechnology in agriculture are made. The selective use of literature, with absence of contradicting research (e.g., Kojima et al. (2004) demonstrated glyphosate lacked affinity for estrogen-α, estrogen-β and androgen receptors) demonstrates consistent and undeterred bias in the authors' publication record. Numerous authoritative reviews have discounted the relevance of the Seralini team's research to human health risk assessment; some of these are referred to in specific publication reviews below. Several more recent publications from this group investigate homeopathic plant extract remedies for effects they attribute to glyphosate exposures in formulated products *in vitro* (Gasnier et al.(2010); Gasnier et al.(2011)).

The key issue with these *in vitro* studies that is that the exposure levels (i.e., body dose) from using Roundup formulations mixed in a spray tank is many orders of magnitude lower than a realistic exposure level. The authors have incorrectly interpreted their exposure levels as representing what workers and perhaps the general public are exposed to. In none of the papers have the authors attempted to use any of the pharmacokinetic data from controlled exposure of rodents or any of the clinical literature to examine whether their cell exposure levels make sense from an environmental perspective. In one of the recent papers (Benachour and Seralini 2009), the authors begin to see some cellular toxicity in a cell culture exposed to a Roundup formulation with a glyphosate dose equivalent to 20 µg/mL. While this concentration seems low to the authors, it is equivalent to a blood level exposure about five times higher than that measured in the blood of rodents after an extreme exposure to an oral dose of 400 mg/kg glyphosate. Under a worst case exposure to an adult female pesticide applicator from all possible exposure routes has been estimated at ~0.125 mg/kg, or 3200 times less than the exposure given to test rodents. Yet, the authors of these cytotoxicity studies did not critically analyze their data in comparison to the 4.6 µg/mL level in blood following the extreme rodent exposure. Furthermore, the authors ignored the fact that skin exposure only results in about 3% absorption of glyphosate in a 24-hour period, and absorption from the intestine is less than 40%. Consequently, the interpretation of in vitro studies is realistic only when concentrations reflect levels likely to occur in blood and/or interstitial fluids.

Another *in vitro* publication claiming a specific developmental toxicity pathway has gained significant public traction, media attention and widespread international public lecture tours by the lead investigator. Paganelli et al. (2010) from the Carrasco research laboratory in Argentina conducted three *in vitro* assays, (i) frog embryos exposed to glyphosate formulation; (ii) frog embryos directly injected without injection blank negative controls; and (iii) fertilized chicken embryos exposed directly to a glyphosate formulation through a hole cut in the egg shell. Key issues surrounding this research include irrelevant routes of exposure as well as excessively high and environmentally unrealistic doses.

In Vivo Glyphosate DART/ED Publications

Relatively few *in vivo* publications on glyphosate DART and ED exist in comparison with the list of *in vitro* publications. Some lack appropriate interpretation of basic toxicology; e.g. Daruich et al. (2001) and Beuret et al. (2005) (two authors are common to each paper and from the same university department) noted rats treated with a glyphosate based formulation showed reduced food intake, reduced water intake and reduced body weight gains. However, the authors did not consider attributing the effects of altered enzyme concentrations to dehydration or restricted diets. Both studies are reviewed in Williams et al. (2012).

Dallegrave et al. (2003; 2007) published results of two non-guidelines rat developmental toxicity studies, in which a glyphosate based formation containing POEA was evaluated. Numerous reporting deficiencies and inconsistencies pose difficulties in data interpretation

Romano et al. (2010) evaluated a glyphosate based formulation in a male pubertal-like assay in Wistar rats, reporting decreased preputial separation, reduced seminiferous epithelial height, increased luminal diameter of seminiferous tubules, and increased relative testicular and adrenal weights. Given the gravity of the reported findings in this publication, a very detailed review was undertaken by experts in the fields

of reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc L and included in Appendix K. Most recently, Romano et al. (2012) reported additional findings in male rats after supposed *in utero* and *post natal* exposures which include "behavioral changes and histological and endocrine problems in reproductive parameters and these changes are reflected by a hypersecretion of androgens and increased gonadal activity, sperm production and libido". As in their first publication, Romano et al. (2012) base their hypothesis on selectively discussed literature implicating glyphosate as an endocrine disruptor, predominantly with citations to research from the Seralini laboratory.

Recently, the first publicly data available from the glyphosate Tier 1 assays under the US EPA Endocrine Disruptor Screening Program, were reported at the 2012 Society of Toxicology meeting (Saltmiras et al., 2012) for the Hershberger and Uterotrophic assays. No effects were noted for any potential for glyphosate to interact with androgenic or estrogenic pathways under these GLP studies following the US EPA 890 Series Test Guidelines.

POEA DART Studies in Williams et al. (2012)

Polyethoxylated alkylamine (POEA) surfactants are a class of non-ionic surfactant, containing a tertiary amine, an aliphatic group of variable carbon chain length and two separate sets of ethoxy (EO) chains of variable length. A dietary exposure assessment of POEAs previously submitted by Monsanto to BfR (Bleeke et al. 2010) is referenced in Doc L and included in Doc K. Williams et al. (2012) recently evaluated and detailed the results of DART studies with two different POEA surfactants, summarized below.

Pregnant female rats were administered MON 0818, a POEA surfactant, at 0, 15 100 and 300 mg/kg/day. The NOAEL for maternal toxicity was 15 mg/kg/day and the NOAEL for rat developmental toxicity was the highest dose tested, 300 mg/kg/day (Holson, 2001).

A reproductive and developmental multigenerational screening study dosed MON 0818 in diets at 0, 100, 300 and 1000 ppm. The majority of endpoints evaluated were unaffected by treatment, including testis morphology, sperm parameters and testosterone and thyroid hormone levels. The mid-dose of 300 ppm (approximately 20 mg/kg/day) was considered the NOAEL for reproductive and developmental toxicity based on the following results in F0 at the high dose, 1000 ppm: increases in unaccounted for implantation sites with reduced mean number of pups and litter size in the high dose group; three high dose dams delivered litters of two-four pups each, with total litter loss by post natal day (PND) 4 in two of these litters. Upon breeding of F1 generation none of the findings noted in F0 were reproducible, and given some were not statistically significant, they were considered equivocal. However, a clear NOAEL for reproductive/developmental toxicity was considered to be the mid dose of 20 mg/kg/day (Knapp, 2007).

Another reproductive/developmental study of a different POEA surfactant, MON 8109 evaluated doses of 0, 30, 100, 300 and 2000 ppm in diet. A single dose group of MON 0818 at 1000 ppm in diet was also included to determine whether litter effects previously noted at this dose were treatment related (Knapp, 2008).

- MON 0818 dosed at 1000 ppm (76 and 86 mg/kg/day premating in males and females respectively) did not reveal the litter effects noted in the previous study at this dose. Two maternal incidents were not considered related to treatment; one female with dystocia died on PND 1 (this was also noted in one female of the control group F1 in the previous study at the same facility) and a second female was euthanized due to a ruptured uterus on gestation day 30. No test substance-related effects were noted for systemic toxicity, reproductive endpoints, pup survival or mortality. Therefore the overall DART NOAEL for MON 0818 was considered 1000 ppm, approximately 81 mg/kg/day.
- The MON 8109 systemic toxicity NOAEL in males and females was 300 ppm, based on mean body weight loss, reduced mean body weight gain and decreased food consumption at 2000 ppm.

Developmental/reproductive effects at 2000 ppm included reduced mean number of implantation sites, increased number of unaccounted for implantation sites, decreased mean litter size at PND 0, reduced mean number of births, reduced survival at PND 4 and reduced mean pup weight at PND 1. The MON 0818 reproductive/developmental NOAEL was also 300 ppm (approximately 23 mg/kg/day).

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Epidemiology Glyphosate DART/ED Publications

Several epidemiology studies in which glyphosate exposure was considered have evaluated the following range of reproductive outcomes; miscarriage, fecundity, pre-term delivery, gestational diabetes mellitus, birth weights, congenital malformations, neural tube defects, attention-deficit disorder / attention-deficit hyperactive disorder (ADD/ADHD). In most instances, glyphosate and reproductive outcomes lack a statistically significant positive association, as described in a recent review of glyphosate non-cancer endpoint publications by experts in the field of epidemiology, Pam Mink, Jack Mandel, Jessica Lundin and Bonnielin Sceurman (Mink et al., 2011). In evaluating ADD/ADHD a positive association with glyphosate use was reported by Garry et al (2002), but cases were parent reported with no clinical confirmation and the reported incidence rate of approximately 1% for the study population was well below the general population incidence rate of approximately 7%. Regarding *in utero* exposures, McQueen et al. (2012) report very low measured dietary exposures, from 0.005% to 2% of the current glyphosate ADI in Europe. Given the low perfusion rate of glyphosate across the placenta (Mose et al., 2008), human *in utero* exposures would be very limited.

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IN VITRO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Walsh, L.P. McCormick, C. Martin, C. Stocco, D.M.	2000	Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives Volume: 108 Number: 8 Pages: 769-776

Abstract*

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Because testicular Leydig cells play a crucial role in male reproductive function by producing testosterone, we used the mouse MA-10 Leydig tumor cell line to study the molecular events involved in pesticide-induced alterations in steroid hormone biosynthesis. We previously showed that the organochlorine insecticide lindane and the organophosphate insecticide Dimethoate directly inhibit steroidogenesis in Leydig cells by disrupting expression of the steroidogenic acute regulatory (StAR) protein. StAR protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. In the present study, we screened eight currently used pesticide formulations for their ability to inhibit steroidogenesis, concentrating on their effects on StAR expression in MA-10 cells. In addition, we determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3 β-hydroxysteroid dehydrogenase (3 β-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl $[(Bu)_2]$cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Ammo, Ambush, Fusilade, Cyclone, Roundup, Banvel, Cotoran, Dual, glyphosate. Surfactants not identified or quantified in formulations.

Active substance(s):
- Ammo: **cypermethrin**: (*R,S*)-α-cyano-3-phenoxybenzyl(1*R,S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Ambush: **permethrin**: 3-phenoxybenzyl(1*R,S*)-*c i s* ,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Fusilade: **fluazifop-*p*-butyl**: (*R*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid
- Cyclone: **paraquat**: 1,1´-dimethyl-4,4´-bipyridinium
- Roundup: **glyphosate**: *N*-(phosphonomethyl) glycine
- Banvel: **dicamba**: 3,6-dichloro-*o*anisic acid
- Cotoran: **fluometuron**: 1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl) urea

	• Dual: **metolachlor**: 2-chloro-6´ethyl-*N*-(2-methoxy-1-methylethyl)aceto-toluidine.
Purity:	• Ammo (300 g/L cypermethrin) • Ambush (240 g/L permethrin) • Fusilade (120 g/L fluazifop-*p*-butyl) • Cyclone (240 g/L paraquat) • Roundup (180 g/L glyphosate) • Banvel (480 g/L dicamba) • Cotoran (480 g/L fluometuron) • Dual (958 g/L metolachlor)
Source:	Glyphosate – Sigma Other pesticides – unknown source

2. Vehicle and/or positive control:

Vehicle control:	Yes (DMSO, ethanol < 0.4 %)
Positive control:	No data

3. Test system / cells / animals:

Cell culture:	Mouse MA-10 Leydig tumor cell line
Species:	Mouse
Source:	M. Ascoli, University of Iowa College of Medicine (Iowa City, IA)
Maintenance conditions:	Waymouth´s MB 752/1 medium + 15% horse serum Temperature: 37° C, Atmosphere: 5% CO_2
Plate cultures #1:	75,000 cells/well in a 96-well plate. For dose–response, time–course, steroidogenic enzyme activity, reversibility, and mixture studies.
Plate cultures #2:	50 x 10^6 cells onto 25 x 25 cm tissue culture dishes.For nuclear run-on analysis.
Plate cultures #3:	1.5 x 10^6 cells into 100-mm culture dishes, grown until 80% confluence.

4. Test methods:

Study type:	Inhibition of steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	2 or 4 h
Dose/concentration levels:	Ambush, Ammo: 5, 10, 50 µg/mL Banvel, Cotoran, Dual, Fusilade: 1, 5, 10 µg/mL Cyclone: 0.5, 1, 5 µg/mL Roundup: 12.5, 25, 50, 100 µg/mL
Treatment:	MA-10 cells were stimulated using a maximal stimulatory dose of $(Bu)_2$cAMP (1 mM). In some tests (P450scc and 3β-HSD enzyme activity), steroidogenic substrates (22R-HC, 25 µM or pregnenolone, 10 µM) were provided. All treatments were performed in serum-free media. Final concentrations of the solvents DMSO and ethanol were < 0.4 %.

5. Observations/analyses:

Dose–response and time-course

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studies:

Measurement: Steroid levels and total protein synthesis.

Calculation: IC_{50} values (concentration that leads to an inhibition of 50%) were calculated as the slope of the linear regression line obtained from Eadie/Hofstee plots of steroidogenesis dose–response data.

Analysis: For steroid determination in Roundup-treated cells, each data point was the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

For progesterone production in cells treated with other pesticides, each data point is the mean ± SE of four replicates in a single experiment that was repeated once.

Progesterone production and total cellular protein synthesis

Radioimmunoassay (RIA).

Measurement: Quantification of progesterone

Preparation of samples: Standard curves were prepared in serum-free Waymouth's medium.

Analysis: Analysis of RIA data was performed using a computer program specifically designed for this purpose (not further specified).

Data are expressed as ng/mL media.

Determination of total cellular protein synthesis:

Measurement: Total protein content was determined using a modification of the Bradford method (no treatment with Expre$^{35}S^{35}S$).

Preparation of samples: After treatment, cells were solubilized in 0.25 M NaOH at 37°C. Protein was precipitated overnight at 4°C using cold 20% trichloroacetic acid (TCA). TCA-precipitable material was transferred onto glass fiber filters, rinsed with 5% TCA, dried, and counted in a liquid scintillation counter.

Analysis: Results were reported as counts per minute per mg protein (2 or 4 h).

Each data point is the mean ± SE of four replicates in a single experiment, which was performed three times.

Determination of P450scc and 3β-HSD activity and reversibility:

Measurement: <u>P450scc enzyme activity:</u> Pregnenolone in medium
<u>3β-HSD enzyme activity:</u> Progesterone in medium

Preparation: <u>Evaluation of P450scc enzyme activity</u>:
22*R*-HC was provided as substrate to MA-10 cells in the presence and absence of the xenobiotic as well as cyanoketone and SU 10603 (inhibitors of 3β-HSD and P450c17, respectively).
<u>Evaluation of 3β-HSD enzyme activity:</u>
pregnenolone was provided as substrate, and MA-10 cells were treated in the presence and absence of the xenobiotic

Analysis: Each data point represents the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

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Effects on enzyme and StAR expression: Protein levels, mRNA levels, gene transcription

Isolation of mitochondria and Western blot analysis:

Measurement: Protein levels of P450scc, β-HSD, StAR

Preparation: Western blot analysis of mitochondrial protein was performed. Mitochondria were isolated by homogenization of the cells followed by differential centrifugation. After detection of StAR, membranes were stripped and then successively probed with P450scc or 3β-HSD antisera.

Analysis: The bands of interest were quantitated using a BioImage Visage 2000 imaging system. Values obtained were expressed as integrated optical density units. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Isolation of RNA and Northern blot analysis:

Measurement: mRNA levels of of P450scc, β-HSD, StAR

Preparation: Total RNA was isolated using Trizol Reagent and quantitated. For Northern blot analysis 20 µg total RNA was loaded into each well. Labeling of cDNA probes for mouse StAR, P450scc, 3β-HSD, and 18S rRNA was achieved by random priming (Prime-It II; Stratagene, La Jolla, CA) using [α-32 P] dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer's protocol. After Northern blot analysis with StAR cDNA, blots were stripped and then successively probed with P450scc, 3β-HSD, and 18S rRNA cDNA.

Analysis: The bands of interest (RNA) were quantified. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Gene expression:

Measurement: StAR, P450scc

Isolation of nuclei:

Preparation: After treatment, cells were harvested with a rubber policeman and centrifuged. The cell pellet was resuspended and homogenized. The homogenate was layered and centrifuged. The supernatant was discarded and the pellet containing nuclei was resuspended, frozen on dry ice, and stored in liquid nitrogen.

Nuclear run-on analysis:

Measurement: Radioactivity was detected using a Phosphorimager 445 SI.

Analysis: Signals were quantitated using ImageQuant version 4.1 software in volume mode, which integrates the intensity of each pixel within the defined area.

Values were obtained as arbitrary units. Each data point represents the average ± SE of five separate experiments.

Protein kinase A (PKA) activity determination:

Measurement: PKA activity was measured with the SignaTECT cAMP-dependent protein kinase assay system.

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Analysis: Three separate experiments were performed in which treatments were performed in triplicate.

Mixture studies:

Measurement: Progesterone was measured.

Analysis: Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Statistics: Statistically significant differences were determined by one-way analysis of variance and Fisher–protected least-square difference multiple comparison using the software program Statview SE + Graphics.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions – Not reliable for Roundup**

Comment: Non-standard test systems, but publication meets basic scientific principles. However, surfactant blend in Roundup confounds results.

2. Relevance of study: **Relevant with restrictions:** Different effects of glyphosate alone and glyphosate formulations were observed. No conclusion can be drawn that the observed effects are result of glyphosate exposure. Roundup data unreliable for endpoints measured, due to mitochondrial membrane damage.

3. Klimisch code: **2 for glyphosate data, 3 for Roundup data**

Response - GTF

- Glyphosate did not affect steroidogenesis in the test system.
- Roundup formulation data was confounded by mitochondrial membrane damage, attributable to the surfactant in the tested formulation.
- Roundup results were comprehensively addressed in Levine et al. (2007).
  - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have "indistinguishable" dose response curves for reductions in progesterone production in hCG stimulated MA-10 Leydig cells. Therefore the effect on progesterone levels shown by Walsh (2000) were independent of glyphosate and attributable to the surfactant component of the formulation.
  - Comparable rates of progesterone inhibition for several different surfactants suggest a common mode of action for surfactants.
  - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have almost identical concentration-dependent decreases in MTT activity in MA-10 cells, suggesting the surfactant alone was responsible for the observed cytotoxicity and effect on mitochondrial function.
  - The JC-1 assay demonstrated the decreased progesterone production in MA-10 Leydig cells was accompanied by loss of mitochondrial membrane potential. These results confirm StAR protein function and steroidogenesis require intact mitrchondrial membrane potential.
  - StAR protein expression were not affected by treatments, indicating that perturbed mitochondrial membrane, not StAR protein inhibition, was responsible for the effects noted by Walsh et al. (2000).

Given the significant differences in physico-chemical properties between glyphosate and formulation surfactants, environmental fate and transport of these compounds are likely to be different. Likewise,

absorption, distribution, metabolism and excretion (ADME) differences between glyphosate and formulation surfactants at low concentration exposures in the field, environment or food residues will very likely result in insignificant concomitant physiological exposures.

References

Levine SL, Han Z, Liu J, Farmer DR, Papadopoulos V. (2007). Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. Cell Biol Toxicol. 23:385-400..

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Author(s)	Year	Study title
Paganelli, A., Gnazzo, V., Acosta H., Lopez, S.L., Carrasco, A.E.	2010	Glyphosate-Based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signalling Chemical Research in Toxicology Volume: 23 Pages: 1586-1595

Abstract*

The broad spectrum herbicide glyphosate is widely used in agriculture worldwide. There has been ongoing controversy regarding the possible adverse effects of glyphosate on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosatebased herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate in development. *Xenopus lae*V*is* embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior-posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (Shh) signaling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The direct effect of glyphosate on early mechanisms of morphogenesis in vertebrate embryos opens concerns about the clinical findings from human offspring in populations exposed to GBH in agricultural fields.
* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Classic ®; Glyphosate
Active substance(s):	Glyphosate, Roundup Classic ®: Monsanto
Source:	Glyphosate: Sigma Aldrich
Purity:	Roundup Classic ®: 48% (w/v) glyphosate salt
	Glyphosate: not reported

2. Positive control: Specified under the respective test

3. Test organisms and systems:

Species:	*Xenopus laevis*
Embryo culture:	*Xenopus laevis* embryos obtained by in vitro fertilisation
Source:	Not specified
Culture conditions:	Embyos were incubated in 0.1 x modified Barth´s saline (MBS)
Species:	Chicken

Strain: White Leghorn

Source: Not specified

Stage: Egg (fertilized)

Guideline: Non-guideline tests

GLP: No

Guideline deviations: Not applicable

***Xenopus* embryo Culture and Treatments:**

Stage of embryos: 2 cell

Dose levels: 1/3000, 1/4000, and 1/5000-dilutions of Roundup Classic® prepared in 0.1× MBS (modified Barth's saline)

Treatment: Treatments were performed from the 2-cell stage.

Rescue experiments: 0.5 or 1 µM Ro-415253 was added at the 9-cell stage

Culture conditions: Embryos were incubated in 0.1 x MBS. Cyclopamine was used at 100 µM concentration in 0.1 x MBS and was applied from the 2-cell stage until fixation. Embryos were fixed in MEMFA when sibling controls reached the desired stage.

Negative control: Not adequately described

Positive control: None

Xenopus Embryo Injections, Whole Mount in Situ Hybridization and Cartilage Staining:

Dose levels: 360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757).

Exposure route: injection

Stage of embryos: 2 cell

Treatment: Embryos were injected with 360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757) per cell into one or both cells at the 2-cell stage. Glyphosate was coinjected with 10 ng of Dextran Oregon Green (DOG, Molecular Probes) to identify the injected side.

Culture condition: Embyos were incubated in 0.1 x MBS. And fixed in MEMFA when sibling controls reached the desired stage.

In situ hybridisation: Wholemount in situ hybridisation (WMISH) was performed with digoxigenin-labeled antisense RNA probes, but without the proteinase K step. Embryos were fixed in MEMFA at stages 45-47, washed with PBS, stained overnight in 0.04 %Alcian Blue, 20% acetic acid, and 80 % ethanol. Afterwards embryos were washed.

Detection of RA Activity:

Dose levels: 1/3000, 1/4000, and 1/5000 Roundup Classic® dilutions

Exposure route: injection

Stage of embryos: 1-2 cell

Treatment: Embryos were injected with 320 pg of the plasmid RAREhplacZ (RAREZ) per cell into one cell at the 2-cell stage and placed immediately in the test substance dilutions

Negative control: Negative control was not evaluated with vehicle injection. Therefore effects of decreased pH or vehicle coformulant (Dextran Orange Green) were not assessed.

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Positive control:	Xenopus embryos were injected with the RAREZ plasmid and incubated at late blastula stage with 0.5 or 5 µM all-transretinoic acid (RA, Sigma R2625).
Rescue experiment:	Embryos injected with the reporter plasmid were incubated in a 1/4000 test substance dilution from the 2-cell stage, and when they reached the blastula stage, 1 µM of Ro 41-5253 was added.

Treatments of Chicken Embryos:

Stage:	Egg
Dose levels:	20 µL of 1/3500 or 1/4500 dilutions of Roundup Classic®.
Treatment:	Injection after opening a small window in the shell of fertilized chicken eggs, above the air chamber in the inner membrane. After injection the window was sealed with transparent adhesive tape
Negative control:	Injected with 20 µL of H2O without pH or osmolality adjustment
Positive Control	None
Pre-incubation conditions:	Placement: eggs were placed with their blunt end up; Temperature: room temperature; Duration: 30 minutes.
Incubation conditions:	Light: Darkness; Temperature: 38°C; Humidity: 56-58% Rotation: regular

Whole-Mount Inmunofluorescence and WMISH of Chicken Embryos:

Treatment:	Embryos were fixed 2-4 hin freshly prepared 4% paraformaldehyde, rinsed and processed for analysis. Wholemount in situ hybridization (WMISH) was performed as described for Xenopus embryos, using a c-shh probe.

4. Measurements/analyses:

Measurements:	Basal luminiscence was detected in uninjected and untreated embryos. The endogenous RA activity was measured in embryos injected with RAREZ (plasmid RAREhplacZ). When sibling controls reached the neurula stages, all embryos were processed for chemiluminiscent quantitation of the reporter activity by using the β-gal reporter gene assay (Roche). Luminiscence was measured on duplicate samples in FlexStation 3 equipment (Molecular Devices), and values were normalized by protein content.
Statistics:	A two-tailed t-test was employed to analyze the significance in the difference of the means. The experiment was repeated three times.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

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Comment: Non-guideline study that is not sufficiently described for assessment. Inadequate positive and negative control experiments.

2. Relevance of study: **Not relevant:** Irrelevant routes of exposure and inappropriately high doses. Test system not adequate for human risk assessment.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- No pH adjustment for doses and thus effects may be in response to the acidic nature of glyphosate technical acid.
- Inappropriate and irrelevant routes of exposure.
- Data requires further substantiation before consideration in risk assessment.

Response 2 – Saltmiras et al. (2012) letter to the Editor

- Multiple high quality toxicological studies and expert review panels consistently agree glyphosate is not a teratogen or reproductive toxicant.
- The authors' justification for this research is flawed, providing no valid basis, other than an opinion, of an increase in the rate of birth defects in Argentina.
- Direct injection of frog embryos and through chicken shells do not reflect real world exposure scenarios to either environmental species or humans.
- Doses were excessively high and irrelevant for risk assessment purposes. Frog embryos were also bathed in glyphosate formulation at doses 9-15 times greater than the acute LC50 same species of frog. Calculating equivalent oral doses based on pharmacokinetics studies, such doses are 150000000 times greater than worst case human exposure monitoring data.
- "…. the results from this research cannot be used in isolation to reach the conclusions expressed in the publication. Instead, the type of data in this research paper must be interpreted relative to all other available data on the specific materials under study and with balanced consideration for higher tier apical studies."

Response 3 – Mulet (2012) letter to the Editor

- Notes the premise for this research is falsely based on an incorrectly cited local pediatric bulletin from Paraguay.
- "…. this article refers to a study in a single hospital in Paraguay showing a correlation between pesticide use (not herbicides as mentioned by Paganelli et al.) and birth malformations. In the cited study (Benitez et al.), the authors state that the results are preliminary and must be confirmed. Is important to remark that the Benitez et al. study does not include any mention to glyphosate, so does not account for what the authors are stating in the Introduction…..This journal is also wrongly cited in the Discussion referring to increased malformations due to herbicides, which is not the result of the study."

Response 4 – comments from BVL (2010)

- Highly artificial experimental conditions.
- Inappropriate models to replace validated mammalian reproductive and developmental toxicity testing methods for use in human health risk assessment.
- Inappropriate routes of exposure.
- Lack of corroborative evidence in humans.
- "In spite of long-lasting use of glyphosate-based herbicides worldwide, no evidence of teratogenicity in humans has been obtained so far."

Response 5– comments from European Commission Standing Committee on the Food Chain and Animal Health (2011)

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- The EU commission supports the German Authorities position, "that that there is a comprehensive and reliable toxicological database for glyphosate and the effects observed have not been revealed in mammalian studies, nor evidenced epidemiologically in humans."
- "…. the Commission does not consider there is currently a solid basis to ban or impose specific restrictions on the use of glyphosate in the EU."

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Author(s)	Year	Study title
Richard, S. Moslemi, S. Sipahutar, H. Benachour, N. Seralini, G.E.	2005	Differential effects of glyphosate and roundup on human placental cells and aromatase. Environmental Health Perspectives Volume: 113 Pages: 716-720

Abstract*

Roundup is a glyphosate-based herbicide used worldwide, including on most genetically modified plants that have been designed to tolerate it. Its residues may thus enter the food chain, and glyphosate is found as a contaminant in rivers. Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned. Here we show that glyphosate is toxic to human placental JEG3 cells within 18 hr with concentrations lower than those found with agricultural use, and this effect increases with concentration and time or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. We tested the effects of glyphosate and Roundup at lower nontoxic concentrations on aromatase, the enzyme responsible for estrogen synthesis. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Active substance(s):	Glyphosate
Source of test item:	Glyphosate: Sigma-Aldrich, Saint Quentin Fallavier, France
Lot / Batch #:	Not specified
Purity:	not reported
Test item:	Roundup ®
Active substance(s):	Glyphosate
Source of test item:	Roundup®, (produced by Monsanto, obtained from a commercial source)
Lot / Batch #:	Not specified
Purity:	Roundup ®: 360 g/L acid

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Cell line:	Human choriocarcinoma derived placental cell line (ref JEG3, ECACC 92120308)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)
Maintenance medium:	Phenol red–free EMEM containing 2 mM glutamine, 1%

nonessential amino acids, 100 U/mL antibiotics (mix of penicillin, streptomycin, and fungizone), 1 mM sodium pyruvate, and 10% fetal calf serum

Cells: Human placental microsomes
Equine testicular microsomes

Source: Human:
Full-term placentas of young healthy and non-smoking women (Centre Hospitalier Régional de Caen, France) and equine testis by differential centrifugations.
Equus:
Equine testis

Microsome preparation: Microsomal fractions (endoplasmatic reticulum) were obtained using differential centrifugations.
Tissues were washed with 0.5 M KCl, homogenised in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM DTT, and centrifuged at 20,000 g. The supernant was ultracentrifuged at 100,000 g, and the pellet was washed twice, disoolved in the same buffer containing 20% glyceol and stored at -70°C until use. All preparations steps were carried out at 4°C.

4. Test methods:

GLP: No (for all tests)

MTT assay: Assessment of cell viability
Cleavage of MTT into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase, to evaluate JEG3 cell viability exposed to Roundup or glyphosate during various times.

Guideline: Non-guideline assays

Guideline deviations: Not applicable

Test substance preparations: 2% solution of Roundup and an equivalent solution of glyphosate were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France), and the pH of glyphosate solution was adjusted to the pH of the 2% Roundup solution (~ pH 5.8). Successive dilutions were then obtained with serum-free EMEM.

Dose concentrations: In serum-containing medium (18, 24, 48 h):
Roundup: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 %
Glyphosate: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 %
In serum-free medium:
Roundup (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %
Glyphosate (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %
Glyphosate + Roundup 0.02% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %
Glyphosate + Roundup 0.1% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %

Treatment: Fifty thousand cells per well in 24-well plates were grown to 80% confluence, washed with serum-free EMEM and exposed to various concentrations of Roundup or equivalent glyphosate concentrations

Incubation conditions: Cells were washed with serum-free EMEM and incubated with 250 µL MTT per well for 3 h at 37°C.

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	250 µL of 0.04 N-hydrochloric acid–containing isopropanol solution was added to each well.
Positive control:	None
Negative control:	None
Replicates per dose level:	3 x 3
Radioimmunoassay (RIA):	Measurement of aromatase activity *in vitro*
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Replicates per dose level:	3 x 3
RT-PCR:	Quantification of cytochrom P450 aromatase mRNA levels in JEG3 cells
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Sample preparation:	Total RNA was isolated from JEG3 cells using the guanidium/phenol/chloroform method. RNA samples were treated with DNase I at 37 C for 30 min to remove genomic DNA. Then DNase I was inactivated at 65°C for 10 min.
Tritiated water release assay:	Assessment of aromatase activity in human placental microsomes *in vitro*
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	Roundup: 0.01, 0.06, 0.1, 0.5, 0.7, 1.0 , 3.0, 6.0 % Glyphosate: 0.01, 0.06, 0.1, 0.7, 1.0 , 3.0 %
Positive control:	None

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Negative control: None

Treatment of human microsomal fractions: 50 µg of human placental microsomes were incubated with radiolabeled androstenedione (100 pmol/tube) at 37°C for 15 min in the presence or absence of various concentrations of Roundup or glyphosate in 1 mL total volume of 50 mM Tris-maleate buffer (pH 7.4).The reaction was started by adding 100 µL of 0.6 mM H±NADPH and stopped with 1.5 mL chloroform and then centrifuged at 2,700 g at 4°C for 5 min. After adding 0.5 mL 7% charcoal/1.5% dextran T-70 solution into the preparation, the centrifugation was repeated fro 10 min.

Treatment of equine microsomal fractions: 2 µg of equine testicular microsomes were incubated for 3 min at 25°C with various concentrations of radiolabeled androstenedione (in the presence or absence of various concentrations of Roundup in 0.5 mL of H^+-NADPH containing Tris-maleate buffer (pH 7.4).

Spectral studies: Assessment of reductase and aromatase activities

Guideline: Non-guideline assays

Guideline deviations: Not applicable

Dose concentrations: Roundup: 0.1 %
Glyphosate: 0.0046 %

Positive control: None

Negative control: None

Purification of reductase / aromatase: Equine reductase was obtained after chromatographic separation, by ω-aminohexyl-Sepharose 4B and adenosine 2´, 5´-diphosphate agarose, respectively, hydrophobic interaction and affinity columns.

Equine cytochrom P450 aromatase was purified from equine microsomes, after its separation from reductase, by successive chromatographic steps.

5. Observations/analyses:

MTT assay

Measurements: The optical density was measured using a spectrophotometer at 560 nm for test and 640 nm for reference.

Radioimmuno assay (RIA)

Measurements: The conversion of androstenedione to E1 by the aromatase complex was measured in cell supernatants by radioimmunoassay (RIA).

The aromatase activity was expressed in relation to the protein concentration that was evaluated in cell extracts using bovine serum albumin as standard

RT-PCR

Measurements: Quantitation of mRNA by RT-PCR using M-MLV-RT (Moloney murine leukemia viruse reverse transcriptase).

The absence of DNA contamination in RNA samples was checked in controls without M-MLV-RT.

All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System.

Tritiated water release assay

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Measurements: Microsomal aromatase activity was evaluated by tritiated water release from radiolabeled substrate [1β-3H]-androstenedione. This method based on the stereo specific release of 1β-hydrogen from the androstenedione substrate, which froms tritiated water during aromatisation.

Aromatase activity was determined by measuring the radioactivity of the 0.5 mL aqueous phase.

Spectral studies:

Measurements: Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H+-NADPH at 550 nm for 2 min at 37 C using a Kontron-Uvikon 860 spectrophotometer.
The absorbance of purified equine aromatase in the presence or absence of glyphosate or Roundup was recorded from 375 to 475 nm with a spectrophotometer.

The spectra of aromatase with glyphosate or Roundup alone were subtracted from the incubation spectrum.

Statistics for all tests: All data are presented as the mean ± SE. The experiments were repeated three times in triplicate unless otherwise indicated. Statistically significant differences were determined by a Student *t*-test using significance levels of 0.01 and 0.05.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design is insufficient for risk assessment of real exposure concentrations. Methodological deficiencies (no controls were included). Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactant are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate at non-cytotoxic concentrations in this test system was demonstrated to have no effects on aromatase activity.
- Likewise, did not affect mRNA levels after 18 hours treatment at ≤ 0.1% glyphosate.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.
- The *in vitro* test system is non-validated
- Physiologically irrelevant concentrations tested
- Testing surfactant-like substances in such systems is now recognized to be not valid.

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Response 2 – summarized from the French Ministry of Agriculture and Fish, Committee for Study of Toxicity (2005)

- Major methodological gaps.
- JEG3 cells, a choriocarcinoma human cell line (average of 70 chromosomes vs 46 in normal human cells).
- Concentrations of Roundup used in the various experiments considered to be extremely high.
  - In consideration of limiting factors (oral absorption, 30%; skin absorption, 0.3%; rapid elimination kinetics), such levels would involve considerable human exposure, or several dozen liters of Roundup diluted at 2%.
  - concentrations of Roundup that trigger an effect on aromatase (0.5% - 2%) are at least 1000 times more effective than those of known aromatase inhibitors, such as azole derivatives
- Study design does not make it possible to show the influence of the adjuvants, nor synergism of adjuvants and glyphosate.
- Multiple non-specific effects of surfactant agents on a broad range of cellular targets not discussed.
- No comparison with comparable surfactant agents intended for household use.
- multiple instances of bias in its arguments and its interpretation of the data.
- The authors over-interpret their results in the area of potential health consequences for humans (unsuitable references, non-sustained in vitro-in vivo extrapolation, etc.).

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Author(s)	Year	Study title
Benachour, N. Sipahutar, H. Moslerni, S. Gasnier, C. Travert, C. Seralini, G. E.	2007	Time- and dose-dependent effects of roundup on human embryonic and placental cells. Archives of Environmental Contamination and Toxicology Volume: 53 Pages: 126-133

Abstract*

Roundup® is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce®) on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD_{50}) Of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2-4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1-2%, i.e., with 21-42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1-2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 µM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

* Quoted from article

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MATERIALS AND METHODS

Cytotoxicity assay

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Antwerp, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce® 9800036

2. Vehicle: Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France)

3. Test system / cells:

Cell cultures: Human embryonic kidney (HEK) 293 cell line (ECACC 85120602)
choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)

Species: Human

Source: CERDIC (Sophia-Antipolis, France)

Cell line maintenance: phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (mix of penicillin, streptomycin, and fungizone), and 10% fetal calf serum (Biowhittaker, Gagny, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate.

Culture conditions: Temperature: 37°C
Atmosphere: 5% CO_2, 95% air
48 h

4. Test method:

MTT assay: Assessment of cell viability

Guideline: None guideline assay

GLP: No

Guideline deviations: Not applicable

Plate culture: 24-well plates, washed with serum-free EMEM

Test conditions: A 2% solution of Roundup and an equivalent solution of glyphosate were prepared in EMEM and the pH was adjusted to about 5.8. From these stock solutions successive solutions were prepared in serum-free EMEM or serum-containing EMEM. The assays were conducted in 24-well plates.
HEK 293 cells or JEG3 cells were grown to 80 % confluence, washed with serum-free EMEM and then exposed to various concentrations of Roundup Bioforce ® or the equivalent concentrations of glyphosate, in serum-free or serum-containing EMEM for 1, 24, 48 or 72 h. Afterwards cells were washed with serum-free EMEM and incubated with 250 µL MTT for 3 h at 37°C. per well. Then 250 µL of 0.04 N-hydrochloric acid containing isopropanol were added to each well, the plates were shaken. Measurements were done at 560 nm for test substance wells and at 720 nm for reference wells.

Dose levels: 0.01, 0.05, 0.1, 0.5, 0.8, 1, 2% of Roundup or equivalent concentrations of glyphosate in serum-free EMEM or serum-containing EMEM

Cells per well: 50000

Exposure duration: 1, 24, 48, and 72 h

Replicates per dose level: 9

5. Observations/analyses:

Measurements: Cell viability

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of $p < 0.01$ or $p < 0.05$.

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Aromatase activity inhibition

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Anvers, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce® 9800036
2. Vehicle and/or positive control:	Specified under the respective assays (see below)
3. Test system / cells:	
Cell culture:	HEK 293 cell line (ECACC 85120602)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)
Tissue for microsome preparation #1:	full-term placentas of young healthy and non-smoking women
Species:	Human
Source:	Centre Hospitalier Régional de Caen (France)
Tissue for microsome preparation #2:	Equine testis
Species:	Horse
Source:	Not reported
Microsome preparation:	Human placental and equine testicular microsomes: Tissue preparation was done by differential centrifugations. All steps were conducted at 4°C. Tissues were washed with 0.5 M KC1, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM Dithiothreiol DTT, and centrifuged at 20,000*g*. The supernatant was then ultracentrifuged at 100,000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at -70 C.
4. Test methods:	
Study type:	Measurement of aromatase activity by tritiated water release assay Measurement of reductase a1ctivity in purified reductasee Moieties from equine testicular microsomes
Guideline:	Non-guideline assays
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	<u>Tritiated water release assay</u>: 293 cells were transfected with human aromatase cDNA and exposed to nontoxic concentrations of glyphosate alone or Roundup. Human placental microsomes were incubated with various concentrations of glyphosate alone or Roundup. Reductase activity: Equine testis microsomes or the purified

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reductase moieties were incubated with or without Roundup

Aromatase inhibition:

Equine testicular microsomes were pre-incubated with a saturating concentration (i.e. 11.6%) or without Roundup.

Dose levels: For aromatase activity:

Glyphosate: < 0.2%
Roundup Bioforce®: 1% of product

Test substance solutions were prepared in EMEM (for 293 cells) and in 50 mM Tris-maleate buffer, pH 7.4 or without pH adjustment (microsomes)

In addition for aromatase and reductase activity:

Roundup at IC_{50} (=)

Exposure duration: Tritiated water release assay:

293 cells: 24 h
human placental microsomes: 15 min
Reductase activity:
Equine testicular microsomes: 15 min
Aromatase inhibition (pre-incubation):
Equine testicular microsomes: 30 min

Replicates per dose level: 9

5. Observations/analyses:

Measurements: Aromatase and residual aromatase activity was determined with the tritiated water release assay. Radioactivity of released tritiated water was assessed by liquid scintillation counting.

Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H^+-NADPH at 550 nm for 2 min at 20 C using a Kontron-Uvikon 860 spectrophotometer.

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study report has several reporting deficiencies in the methods section (e.g. test conditions for the pH- and temperature dependent assay not reported). There is no information on the suitability of the used HEK 293 cell line for assessment of hormonal activity. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactant are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – GTF

- Glyphosate at and above relevant concentrations for this test system was demonstrated to have no effects on aromatase activity.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.
- Comparable research to Richard et al (2005), but with an additional cell line, HEK 293, derived from aborted human embryo kidneys, transformed by inserting adenovirus DNA.
- Excessively high doses tested, not environmentally relevant for human health or environmental risk assessment.
- Aromatase production within the steroidogenesis pathway. Therfore, aromatase inhibition would be detected in the steroidogenesis assay. The OECD multi-laboratory validation of the steroidogenesis assay evaluated glyphosate, demonstrating no impact on the steroidogenesis pathway (Hecker et al., 2010).

Response 2 – summarized from Williams et al. (2012)

- pH of test system not adjusted to physiologically appropriate levels
- Negative controls were not pH adjusted to appropriate levels
- Confounding surfactant effects due to cell membrane damage render data generated with formulated products in this test system null.

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Author(s)	Year	Study title
Benachour, N., Seralini, G. E.	2009	Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chemical Research in toxicology Volume: 22 Pages: 97-105

Abstract*

We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup formulations, from 10(5) times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®; AMPA

Active substance(s): Glyphosate

Source of test items: Glyphosate: Sigma-Aldrich, France
Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (produced by Monsanto, all available on the market)

Lot/Batch #: Not specified

Purity: Glyphosate: not reported
Roundup Express®: 7.2 g/L (R7.2)
Bioforce® or Extra 360: 360 g/L (R360)
Grands Travaux®: 400 g/L (R400)
Grands Travaux plus®: 450 g/L (R450)

Homologation: Roundup Express®: 2010321
Bioforce® or Extra 360: 9800036
Grands Travaux®: 8800425
Grands Travaux plus®: 2020448

Test item: AMPA (aminomethylphosphonic acid)

Source: Sigma-Aldrich (Saint Quentin Fallavier, France)

Lot / Batch #: Not reported

Purity: Not reported

Test item: Polyethoxylated tallowamine (POEA)

Source: Pr. R. Bellé (UMR 7150 CNRS/UPMC, Station Biologique de Roscoff, France)

Lot / Batch #: Not reported

Purity: Not reported

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Primary cell culture: HUVEC (human primary cells of the umbilical vein cord endothelial cells)

Source: Lonza

Culture conditions: Specific endothelial growth medium EGM-2 SingleQuots (CC-4176) containing hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), FBS (fetal bovine serum), VEGF, hFGF-B, R3-IGF-1, ascorbic acid, and heparin.

Cells were grown in 48-well plates over a period of 24 h at 37 °C (5% CO_2, 95% air) to a confluence of 80%. Afterwards they were washed with serum-free EGM-2.

Cell lines: Human embryonic kidney 293 cell line (ECACC 85120602)
Human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)

Source: CERDIC (Sophia-Antipolis, France)

Culture conditions: Phenol red-free Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL antibiotics (a mix of penicillin, streptomycin, and fungizone; Lonza), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France), and 10% FBS (PAA, les Mureaux, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate.

50000 cells were grown at 37°C (5% CO_2, 95% air) over a 48 h period to 80% confluence and were washed with serum-free EMEM.

4. Test methods:

MTT assay: Assessment of cell viability

ToxiLight® assay: Bioluminescent assay for quantitative measurement of cell membrane damage

Caspase-Glo® 3/7 assay Assessment of caspase activity or apoptosis induction

Microscopy: Assessment of cell viability due to cell morphology

Guideline: Non-guideline assays

GLP: No

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Guideline deviations: Not applicable

Cell treatments for all tests: Cells were exposed to various dilutions of the four Roundup formulations, glyphosate, AMPA and POEA in serum-free medium for 24 hours.

In another case, cells were incubated with glyphosate, AMPA, and POEA mixtures by pairs at the final nontoxic dilution on SD (succinate dehydrogenase) of 0.5% on the human cell lines (293 or JEG3) and 0.05% on the human primary cells (HUVEC) in comparison to Roundup Bioforce or Extra 360.

Dose levels: Roundup formulations, glyphosate, AMPA and POEA: 14 concentrations ranging from 10 ppm to 2 %
Additional AMPA concentrations: 4, 6, 8 and 10%
POEA concentrations: 1 and 5 ppm

Combined exposures of G, AMPA and POEA mixtures:

For the two cell lines, the first mixture was the combination of glyphosate (0.4999%) with POEA (0.0001%); the second was the combination of glyphosate (0.4%) with AMPA (0.1%), and the third was AMPA (0.4999%) plus POEA (0.0001%).

Combined exposures of G, AMPA and POEA mixtures:

For the primary HUVEC cells, the first mixture was glyphosate (0.04999%) with POEA (0.0001%); the second was glyphosate (0.04%) with AMPA (0.01%), and the third was AMPA (0.04999%) plus POEA (0.0001%).

Test conditions: MTT assay: After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum-free medium and incubated with 200 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards 200 µL of 0.04 N-hydrochloric acid containing isopropanol were added, the plates were shaked. Optical density was measured at 570 nm.

ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated under agitation with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium.

Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptosis induction. After treatment of 50 µL cell cultures to various dilutions of test items as described above, 50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 15 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium. The luminescence was measured using a luminometer at 565 nm.

Cell Microscopy: At the end of the 24 h treatments, the serum-free medium was removed, and cells were fixed in absolute ethanol –chloroform – acetic acid (6:3:1, v/v/v) for 1 day at -

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20°C. Each well was washed with PBS (pH 7.4) and incubated with 1 µg/mL DAPI solution. Staining of DNA with DAPI was examined using a fluorescence microscope.

Replicates per dose level: 3

5. Observations/analyses:

Measurements: Cell viability, membrane damage, apoptosis induction, cell morphology

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactant are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed. No positive controls were included.

2. Relevance of study: **Not relevant** (Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants)

3. Klimisch code: 3

Response – summarized from the French Agency for Food Safety (AFSSA, 2009)

- Cell lines used present characteristics which may be at the source of a significant bias in the interpretation of the results.
- Experiments were conducted with 24 hours exposure in a medium without serum, which could lead to disturbance of the physiological state of the cells.
- The glyphosate used in the study is glyphosate acid, whereas in the preparations tested it is in the form of an isopropylamine salt. No precise information is given about the pH of test concentrations except the highest dose.
- No mention of any positive evidence for the apoptosis test.
- Cytoxicity and induction of apoptosis may due to pH and/or variations in osmotic pressure on cell survival at the high doses tested.
- Surfactant (tensoactive) effects and increased osmolality are known to increase membrane permeability, causing cytotoxicity and induction of apoptosis.
- Conclusions are based on unvalidated, non-representative cell models (in particular tumour or transformed cell lines) directly exposed to extremely high product concentrations in culture conditions which do not observe normal cell physiological conditions.
- No new information is presented on mechanism of action of glyphosate and preparations containing glyphosate.
- The authors over-interpret their results with regard to potential health consequences for humans, based in particular on an unsupported *in vitro–in vivo* extrapolation
- The cytotoxic effects of glyphosate, its metabolite AMPA, the tensioactive POAE and other glyphosate-based preparations proposed by Benachour and Seralini do not add any pertinent new

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facts which call into question the conclusions of the European assessment of glyphosate or those of the national assessment of the preparations.

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Author(s)	Year	Study title
Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., Seralini, G. E	2009	Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology Volume: 262 Number: 3 Pages: 184-191

Abstract*

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERα, ERβ) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

* Quoted from article

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MATERIALS AND METHODS

Cytotoxicity assays

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L (R7.2) Bioforce® or Extra 360: 360 g/L (R360) Grands Travaux®: 400 g/L (R400) Grands Travaux plus®: 450 g/L (R450)

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2, breast cancer cell line MDA-

MB453-kb2

Species: Human

Source: HepG2: ECACC, Salisbury, UK
MDA-MB453-kb2: ATCC, Molsheim, France

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

Culture conditions MDA-MB453-kb2: Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

MTT assay: Assessment of cell viability of HepG2 cells

ToxiLight® assay: Bioluminescent assay for measurement of cell membrane damage of HepG2-cells

Alamar Blue® assay: Assessment of cell viability of HepG2 cells

Caspase-Glo® 3/7 assay Assessment of caspase activity or apoptose induction

Neutral red assay: Assessment of cell viability of MDA-MB453-kb2 cells

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Test conditions: MTT assay: 2% Roundup Bioforce® and an equivalent solution of glyphosate to Roundup Bioforce were prepared in serum-free medium and adjusted to pH 5.8. From these stock solutions consecutive dilutions up to 10^{-7} were used for measurement. Assays were conducted in 48-well plates. After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum free medium and incubated with 120 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards 120 µL of 0.04 N-hydrochloric acid containing isopropanol were added, the plates were shaked. Measurements were done at 570 nm.

ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium.

Alamar Blue assay: About 30000 HepG2 cells per well were grown for 24 h in 96-well plates and then exposed to 250 µL of test substance solutions for 24 h (at pH 7.4). Afterwards 100 µL of Alamar Blue solution was added to each well and incubated for 2 h at 37°C. The optical density was measured at 540 and 620 nM. The viability was expressed as percentage of the control results (medium only).

Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptose induction. Cells were exposed to R450 for 24 or 48 h in 96-well plates. Afterwards

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50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 45 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium.

Neutral red assay: about 50000 MDA-MB453-kb2 cells were seeded in 24-weel plates and grown for 24 h at 37°C. Afterwards cells were exposed to test substance solutions for 24 h. Cells were washed and incubated with neutral red solution for 3 h at 37°C. After a further washing the viability was assessed by fluorescence measurement.

Dose levels: Glyphosate: not reported
Roundup Express®: 7.2 g/L
Bioforce® or Extra 360: 360 g/L
Grands Travaux®: 400 g/L
Grands Travaux plus®: 450 g/L

Replicates per dose level: 4 x 3 replicates

5. Observations/analyses:

Measurements: Cell viability, membrane damage, apoptose induction

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

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__Genotoxicity test__

1. Test material:

Test item: Grands Travaux®

Active substance(s): Glyphosate

Source of test items: Grands Travaux® (available on the market)

Lot/Batch #: Not specified

Purity: 400 g/L

2. Vehicle and/or positive control: medium / Benzo[a]pyrene 50 µM

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2

Species: Human

Source: HepG2: ECACC, Salisbury, UK

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type: Single-cell gel electophoresis assay (Comet assay)

Guideline: Non-guideline assay
The assay was conducted according to the method developed by Singh et al., 1988, with some modifications for cell preparation (Valentin-Severin et al., 2003).

(Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 84–191. Valentin-Severin, I., Le Hegarat, L., Lebon, A.M., Lhuguenot, J.C., Chagnon, M.C., 2003. Use of hepG2 cell line for direct or indirect mutagens screening: comparative investigations between comet and micronucleus assay. Mut. Res. 536, 79-90)

GLP: No

Guideline deviations: Not applicable

Dose levels: 1, 2.5, 5, 7.5, 10 ppm

Exposure duration: 24 h

Replicates per dose level: 3 x 2 replicates

Analysed cells per replicate: 100

5. Observations/analyses:

Measurements: Observed nuclei were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium damage) and 3 (maximum damage)

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

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Aromatase disruption

1. Test material:

Test item: Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®

Active substance(s): Glyphosate

Source of test items: Glyphosate: Sigma-Aldrich, France
Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)

Lot/Batch #: Not specified

Purity: Glyphosate: not reported
Roundup Express®: 7.2 g/L
Bioforce® or Extra 360: 360 g/L
Grands Travaux®: 400 g/L
Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2

Species: Human

Source: HepG2: ECACC, Salisbury, UK

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type: Measurement of aromatase activity by tritiated water release

assay, semi-quantitative RT-PCR

Guideline: Non-guideline assay

GLP: No

Guideline deviations: Not applicable

Test conditions: Tritiated water release assay: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup.

RT-PCR: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup. RNA was extracted and reverse transcribed (using 200 U MMLV-RT at 42°C for 60 min). The resulting cDNA was subjected to RT-PCR.

Dose levels: Glyphosate: 0.06, 0.2, 0.3%
Roundup Express®: 0.3, 0.5, 0.8% of product
Bioforce® or Extra 360: 0.08, 0.1, 0.3% of product
Grands Travaux®: 0.001, 0.003, 0.005 % of product
Grands Travaux plus®: 0.005, 0.007 % of product

Exposure duration: 24 h

Replicates per dose level: 4 x 3 replicates

5. Observations/analyses:

Measurements: Tritiated water release assay: radioactivity of released tritiated water was assessed by liquid scintillation counting.

RT-PCR: Aromatase mRNA levels were normalised with control gene GAPDH and analysed photographically.

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

Anti-estrogenic and anti-androgenic effects

1. Test material:

Test item: Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®

Active substance(s): Glyphosate

Description:

Source of test items: Glyphosate: Sigma-Aldrich, France
Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)

Lot/Batch #: Not specified

Purity: Glyphosate:
Roundup Express®: 7.2 g/L
Bioforce® or Extra 360: 360 g/L
Grands Travaux®: 400 g/L
Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Medium / ICI 182 x 780 (10^{-8}M) and Nilutamide (10^{-6} M)

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2, breast cancer cell line MDA-MB453-kb2

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Species: Human

Source: HepG2: ECACC, Salisbury, UK
MDA-MB453-kb2: ATCC, Molsheim, France

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum
For anti-estrogenic activity, HepG2 cells were grown in phenol red-free MEM

Culture conditions MDA-MB453-kb2: Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

Gene-receptor tests with luciferase activity measurement

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Test conditions: Anti-estrogenic activity test: 120000 HepG2-cells per well were grown at 37°C (5% CO_2, 95% air) in MEM supplemented with 2 mM glutamine, 1% non-essential amino-acis and 10% of dextran-coated charcoal foetal calf serum in 24-well plates. After 24 h the cells were transfected with a mixture of 5 different plasmids (ERE-TK, hERα, hERβ, pCMVβGal and psG5) and incubated for 1 h at 37°C (5% CO_2, 95% air). Afterwards the medium was removed and replaced by1 mL of medium without foetal calf serum and incubated for further 24 h. Cells were co-treated with the test substance solutions and β-estradiol (10^{-8} M). ICI 182 x 780 (10^{-8} M) served as positive control. At the end of treatment cells were lysed with Reporter lysis buffer and frozen at -80°C for at least 30 min, and prepared for activity measurements.

Anti-androgenic activity test: 50000 MDA-MB-453-kb2 cells per well were grown in 24-well plates in L-15 medium without phenol-red supplemented with 5% dextran-charcoal fetal calf serum at 37°C without CO_2. After 24 h the medium was removed and cells were washed with PBS and exposed to Roundup solutions in co-treatment with DHT (4×10^{-10} M). Nilutamide (10^{-6} M) was used as positive control. After 24 h cells were lysed and luciferase activity was measured.

Dose levels: Anti-estrogenic activity test:
Glyphosate: 0.1, 0.2, 0.3%
Roundup Express®: 0.1, 0.2, 0.3% of product
Bioforce® or Extra 360: 0.05, 0.1, 0.15, 0.2% of product
Grands Travaux®: 0.00025, 0.0005, 0.00075, 0.001 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003 % of product

Anti-androgenic activity test:
Glyphosate: 0.05, 0.1, 0.15%
Roundup Express®: 0.05, 0.1, 0.15, 0.2% of product
Bioforce® or Extra 360: 0.01, 0.02, 0.03, 0.04, 0.05% of product

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Grands Travaux®: 0.00005, 0.0001, 0.00015, 0.0002 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003, 0.004 % of product

Replicates per dose level: 3 x 3 replicates

5. Observations/analyses:

Measurements: Anti-estrogenic activity test: Luciferase and β-galactosidase activities and protein level.

Luciferase activity for each treatment group was normalised to β-galactosidase activity and protein level (Luc x Prot/Gal) and compared to the control (17 β-estradiol) set at 100%.

Anti-androgenic activity test: Luciferase activity were measured and reported as a percentage of the data obtained with the androgen DHT

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Due to reporting deficiencies (e.g. correlation between concentration used in toxicity tests and concentrations used in comet assay) assessment of results difficult. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactant are well known for in vitro test systems.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants. Cytotoxicity has confounded measurements of specific biochemical endpoints.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate demonstrated no significant anti-estrogenic potential
- Glyphosate demonstrated some anti-androgenic potential at lower concentrations, but not as doses increased and therefore results are considered unrelated to treatment
- Four glyphosate based formulations demonstrated both estrogenic and androgenic activity.
- Results are confounded due to surfactants within the formulated products tested, which affect cell membrane integrity and produces false findings.

Response 2 – summarized from BfR Review (2009)

- Numerous methodological flaws are noted.
  - Test substance(s) not characterized
  - Source of materials for cell culture not provided.
  - Dosing concentrations not well described
  - Serum free media only appropriate for short term (3-4 hour) *in vitro* exposures.
  - pH control of dilutions not clear.
  - Osmolality of test solutions not reported.

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    - Electrophoresis parameters insufficiently or inaccurately reported.
- Numerous reporting deficiencies are noted.
    - Influence of serum-free cell culturing on endpoints can not be determined
    - Incomplete data reporting, in cluding β-galactosirase activity, cototoxicity for select assays.
    - Positive control data not reported.
    - Confusion between maximum residue levels verses systemic concentrations in humans.

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Author(s)	Year	Study title
Clair, E., Mesnage, R., Travert, C., Seralini, G.E.	2012a	A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels Toxicology in Vitro Volume: 26 Number: 2 Pages: 269-279

Abstract*

The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000 ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48 h of Roundup exposure Leydig cells are damaged. Within 24–48 h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested and only with glyphosate in regulatory tests.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Description:	Not reported
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: not reported
Lot/Batch #:	Not reported
Purity:	Glyphosate: not reported Roundup Bioforce®: 360 g/L acid glyphosate (corresponding to 100%)
Homologation:	Roundup Bioforce® 9800036

2. Vehicle and/or positive control:

Dulbecco Modified Eagle's Medium/Ham F12 Medium (DMEM; Biotech GmbH, Dutscher, Brumath, France)

3. Test system / cells / animals:

Species:	Rat
Strain:	Sprague-Dawley
Source:	Janvier, Le Genest-Saint-Isle, France or University Centre of

Biological Resources, Caen, France

Age of test animals at study initiation: 70 days ± 5

Sex: male

Body weight: Not reported

Acclimation period:: Not reported

Diet/Food: Standard food, *ad libitum*

Water: Water, *ad libitum*

Housing:: Not reported

Environmental conditions: Temperature: 20 ± 22°C
Humidity: not reported
Air changes: not reported
12-hour light/dark cycle

Cell Culture: Leydig, Sertoli and germ cells

Species: Rat

Source: Sprague-Dawley rats

Cell line maintenance: DMEM/Ham F12 nutrient medium (1:1, v/v) supplemented with or without hGC (human homolog of LH physiologically involved in endocrine regulation of Leydig cells) for Leydig cells culture and with serum replacement 3 for Sertoli and germ cells.

Culture conditions:: Temperature: 32°C
Atmosphere: 5% CO_2, 95% air

4. Test methods:

Bioluminescent ToxiLight™ bioassay: Cytotoxicity assessment

Guideline: Non-guideline assay

GLP: No

Guideline deviations: Not applicable

Plate culture: 96 or 24-well plates

Test conditions: Before the assay, cells were treated with different dilutions of Roundup Bioforce ® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The adenylate kinase detection reagent (AKDR) was prepared in a buffer (5 g/10 mL). Subsequently 50 mL of supernatant were transferred to an opaque black 96-well plate. 50 µL of AKDR reagent were put into each well. The plates were then left under agitation for 15 min in the dark, and light was measured using a luminometer.

Dose levels: Not exactly specified; several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium

Cells per well: 10^5 per well in 96-well plates and 3 x 10^5 per well in 24-well plates

Exposure duration: 3, 6, 9, 12, 18, 24 or 48 h

Replicates per dose level: 9

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Caspase –Glo™ 3/7 assay:	Cytotoxicity assessment, apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96 -well plates
Test conditions:	Before the assay, cells were treated with different dilutions of Roundup Bioforce® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The Caspase-Glo® 3/7 reagent was prepared in a buffer. After 30 min at room temperature, 50 µL of Caspase-Glo® 3/7 reagent was added to 50 µL of culture medium containing the cells previously treated. After shaking the plate 15 min, an incubation period of 45 min at ambient temperature in the dark was required to stabilize the signal before luminescence measurement with a luminometer was performed.
Dose levels:	Not exactly specified, several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	10^5 per well in 96-well plates
Exposure duration:	3, 6, 9, 12, 18, 24 or 48 h
Replicates per dose level:	9
DAPI-labelling:	Apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	24 -well plates
Test conditions:	After 24 h incubation with various dilutions of the test substances, 24-well plates were centrifuged and the medium was removed slowly. Leydig cells were fixed for a day in ab absolute ethanol-chloroform–acetic acid (6:3:1, v/v/v) at -20 °C. The wells were rinsed with PBS (pH7.4) and incubated with 1µg/mL of a solution containing DAPI during 30 min. Each well was washed with water and then observed with a microscope using a fluorescent mode.
Dose levels:	0.05, and 1 % of Roundup Bioforce® and 1% of glyphosate in DMEM/Ham F12 medium
Cells per well:	30000 per well in 24-well plates
Exposure duration:	24 h
Replicates per dose level:	9
3β-hydroxysteroid dehydrogenase (3β-HSD) activity:	Assessment of testosterone production
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96-well plates

Test conditions: Leydig cells were exposed to different concentrations of the test substances. Afterwards the wells containing the pretreated cells and 3β-HSD reagent containing DHEA (substrate), NAD (cofactor), NBT and nicotinamide were incubated at 37 °C for 45-60 min**.** Subsequently, as soon as the cells were stained, a solution of 10% acetic acid was added to solubilise the previously formed formazan crystals. The 3β-HSD activity was then measured by reading the optical density of each well at 560 nm (formazan) through a plate reader.

Dose levels: Not exactly specified; several concentrations from 0 – 0.1% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium

Cells per well: Not reported

Exposure duration: 24 h

Replicates per dose level: 9

Radioimmunoassay (RIA) of testosterone: Assessment of testosterone production

Guideline: Non-guideline assay

GLP: No

Guideline deviations: Not applicable

Plate culture: Not reported

Test conditions: The RIA was carried out on Leydig cells by competition and stopped using the method of activated charcoal. 200 µL of unlabeled testosterone standard solution, phosphate buffer or culture supernatant were incubated with 100 µL of radioactive testosterone and 100 µL of rabbit anti-testosterone antibody. After 30 min at ambient temperature the mixture was placed at 4 °C until the next day. Afterwards 500 µL of charcoal/dextran (50%/5%) was added and the mix incubated at 4 °C. Finally, the tubes were centrifuged (10 min at 2400 rpm at 4 °C) and the radioactivity counted.

Dose levels: 0, 0.0001, 0.0005, 0.001, 0.0025, 0.005, 0.0075 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium

Cells per well: Not reported

Exposure duration: 24 h

Replicates per dose level: 9

Real time PCR: Measurement of mRNA expression of aromatase, androgen receptor and estrogen receptor α- and β.

Guideline: Non-guideline assay

GLP: No

Guideline deviations: Not applicable

Plate culture: 6-well plates

Test conditions: After exposure of Leydig cells with the test substances cell pellets were treated with Trizol for the cell degradation. The chloroform was added to recover the aqueous phase containing the RNA. RNA precipitation was done by adding isopropanol and washing by adding 70% ethanol.

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250 ng of RNA , 200 U of MMLV-RT (Moloney murine leukemia virus reverse transcriptase), 0.2 g of random primers, 500 mM of each dNTP and 20 U of recombinant RNasin® were incubate 90min at 37°C to obtain cDNA, The reaction was stopped by 5 min at 75 °C. The polymerase chain reaction was performed on cDNA using the method GoTaq® qPCR Master Mix (Promega). The PCR conditions were an initial step at 95 °C for 3 min, then 40 cycles of 30 s at 95°C abd 60°C for 60 s. mRNA levels of aromatase, estrogen receptor α and β and androgen receptor were normalized using the L19 control gene.

Dose levels: 0, 0.001, 0.005 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium

Cells per well: Not reported

Exposure duration: 24 h

Replicates per dose level: 9

6. Observations/analyses:

Measurements: Citotoxicity of Roundup Bioforce® or glyphosate measured through adenylate kinase activities; measurements of caspases 3 and 7 (key caspases of apoptosis) in cell cultures by means of bioluminescence-based method; study of chromatin condensation by DAPI-labelling; measurement of 3β-HSD activity; changes in testosterone production secreted from Leydig cells in medium

Statistics: All data are present as means ± SEM. Statistically significant differences from controls were determined by an ANOVA test followed by Bonferroni post-test with p<0.001 (****), p<0.005 (***), p <0.01 (**) and p <0.05 (*).

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KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline *in vitro* test with methodological (i.e. no positive controls included) and reporting deficiencies (e.g. dose levels not always specified).

2. Relevance of study: **Not relevant** (Due to reliability. In addition, *in vitro* data, do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment purposes.)

3. Klimisch code: **3**

Response - GTF

This publication presents no new findings relevant to the current discussions of glyphosate safety. It is clear from the previous work of Seralini and others that surfactants can injure or kill cells when applied to exposed cells living in a Petri-dish environment. It also is not surprising that injured cells demonstrate activation of injury-response systems or suffer from a general decline in a wide variety of cellular functions, including hormone production in cells which normally serve that function. The concentrations used in these experiments are not relevant to human exposures to glyphosate and the experimental system used is not relevant to whole animal outcomes. Importantly, the alleged impacts on endocrine function have not been observed in animal studies of glyphosate or other components of glyphosate formulations at relevant concentrations. Authors state that the lowest concentration of glyphosate tested was 50 ppm,

several orders of magnitude higher than an anticipated human intake (based on pharmacokinetics described in Anadon et al., 2009) following worst case dietary exposure at the ADI.
The experiments reported in this publication involve two additional cell types; Leydig and Sertoli cells from rat testes. However, Petri dish experiments in a laboratory are not representative of exposures to a living animal and are not informative about real-world risks to humans. Instead, these experiments demonstrate what we already know – substances, soaps, detergents of surfactants, can injure unprotected cells *in vitro*.

Author(s)	Year	Study title
Hokanson, R., Fudge, R., Chowdhary, R., Busbee, D.	2007	Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate.Human & Experimental Toxicology Volume: 26 Pages: 747-752

Abstract*

Gene expression is altered in mammalian cells (MCF-7 cells), by exposure to a variety of chemicals that mimic steroid hormones or interact with endocrine receptors or their co-factors. Among those populations chronically exposed to these endocrine disruptive chemicals are persons, and their families, who are employed in agriculture or horticulture, or who use agricultural/horticultural chemicals. Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized *in vitro* DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes, determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed *in utero*.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate formulation
Source:	Unknown retail supplier
Purity:	Not reported
Concentration:	15% home use preparation
2. Vehicle and/or positive control:	SFBS medium / no positive control

3. Test system/cells:

Cell line:	MCF-7
Source:	American Type Culture Collection (Rockville, MD, USA)
Growing medium:	MEM (minimal essential medium), phenol red-free MEM
Source:	Gibco (Gaithersburg, MD, USA)
Culture conditions:	Not reported
Further materials:	17β-estradiol (E2) (Sigma, St. Louis, USA), fetal bovine serum (FBS) (Summit Biotechnology, USA) RZPD microarray chips (Deutsches Ressourcencentrum für

Genomforschung GmbH, Berlin, Germany)
Roche´s cDNA synthesis kit (Roche)
Real time PCR kit (ABI, NJ, USA); ABI 7500 Real-Time PCR system thermocycler (ABI, USA)

4. Test method:

Study type: *In vitro* DNA microarray analysis, quantitative real-time PCR (qrtPCR)

Guideline: None

GLP: No

Guideline deviations: Not applicable

Dose levels: 0.1, 0.01, 0.001 or 0.0001% dilutions of the glyphosate stock solution containing 15% glyphosate.

Duration of exposure: 18 h

Exposure: MCF-7 cells were grown in MEM in T-150 vented culture flasks. Upon reaching 60% confluency, the medium was removed and replaced with phenol red-free MEM containing 10% stripped fetal bovine serum (SFBS), to reduce the E2 availability to the cells. After a growing period of 24 hours the cells were treated with glyposate concentrations at 0.1, 0.01, 0.001 or 0.0001% dilutions of the stock solution (i.e. 15% glyphosate) with or without 3×10^{-10} M E2 for 18 hours.

DNA micro array: Microarray analysis was performed in commercially available microarray slides. After 18 h exposure cells were harvested and RNA was purified. Closed DNA (cDNA) was generated from the isolated RNA using Roche´s cDNA synthesis kit. Cyanine-5 and cyanine-3-labeled anti-sense RNA was generated and hybridized using Wellmer´s protocol. The labelled RNA was loaded with a labelled control sample onto the array slides. Array slides were scanned in an Axon Genepix 4000B. Details of the hybridisation and scanning procedures were not reported.

qrtPCR: Test was conducted in semi-skirted 96 well PCR plate using a commercially available PCR system

Replicates per gene of interest: 3

5. Observations/analyses:

Measurements: Scan of microarray slides, quantitative rt-PCR

Statistics: Statistical analysis utilized one-way ANOVA followed by Dunnett's test to analyse differences between control and chemically treated samples, with $P < 0.05$ considered to be statistically significant.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Not acceptable *in vitro* methods for test mixtures containing surfactant. Well documented study publication which meets basic scientific principles, but surfactants are inappropriate test substance in cell lines.

2. Relevance of study: **Not relevant** Temporal altered gene expression is not a biomarker for toxicity, but rather, may be within the range of normal biological responses of homeostasis. *In vitro*

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cytotoxicity of surfactants, however, is a significant confounder in data interpretation. Data do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment purposes.)

3. Klimisch code: **3**

Response - GTF

- Relevance of altered gene expression in a cell line derived from a breast cancer should not be extrapolated to reflect human health endpoints.
- Altered gene expression should not be confused with adverse health outcomes. Rather altered gene expression may equally be considered a biological response within the range of normal homeostasis.
- The authors describe a “bewildering array” of possible human health endpoints, which are conspicuously absent in the vast glyphosate toxicology data base.
- The concluding sentence, with implications of both adult and foetal cell damage, lack biological plausibility when considering glyphosate *in vivo* ADME, kinetics and toxicology data.

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IN VIVO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Daruich, J. Zirulnik, F. Gimenez, M. S.	2001	Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses Environmental Research Volume: 85 Pages: 226-231

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Abstract*

To prevent health risk from environmental chemicals, particularly for progeny, we have studied the effects of the herbicide glyphosate on several enzymes of pregnant rats. Glyphosate is an organo-phosphorated nonselective agrochemical widely used in many countries including Argentina and acts after the sprout in a systemic way. We have studied three cytosolic enzymes: isocitrate dehydrogenase-NADP dependent, glucose-6-phosphate dehydrogenase, and malic dehydrogenase in liver, heart, and brain of pregnant Wistar rats. The treatment was administered during the 21 days of pregnancy, with 1 week as an acclimation period. The results suggest that maternal exposure to agrochemicals during pregnancy induces a variety of functional abnormalities in the specific activity of the enzymes in the studied organs of the pregnant rats and their fetuses.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Herbycigon
Active substance(s): Glyphosate
Source: Herbycigon: M.F.L. S.R.L., Argentinia
Lot/Batch #: Not reported
Purity: Not reported

2. Vehicle: Tap water

3. Test animals:

Species: Rat
Strain: Wistar
Source: National University of San Luis, Argentina
Age of test animals at study initiation: Not reported
Sex: Females
Body weight: 210-230 g
Acclimation period: 1 week
Diet/Food: 20 g of stock laboratory diet (elaborated at Cargill) per day; ingredients: meat flour, bone and meat flour, fish meal, blood flour, soybean meal, toasted soybean, soy expeller, sunflower flour, cotton flour, peanut meal, animal fat, corn, wheat, sorghum, oat, barley, wheat bran, rice bran, gluten meal, vitamins A, E, B, D3, K3, and B12, niacin, pantothenic acid, choline, ascorbic acid, bone ash, salt, calcium carbonate, oyster, manganese oxide, zinc oxide, ferrous sulfate, copper

	oxide, sodium selenite, iodine, and cobalt.
Water:	35 ml of potable water per day
Food and water for control group:	Low water and food (10 ml and 10 g, respectively)
Housing::	After mating, individually in cages
Environmental conditions:	Temperature: 22-25°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Enzymatic activity of cytosolic enzymes in pregnant rats and fetuses
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	21 days during pregnancy
Dose levels:	0 (tap water), glyphosate solution 0.5% w/v in tap water (dose: 0.2 ml glyphosate/ml water), glyphosate solution 1% w/v in tap water (dose: 0.4 ml glyphosate/ml water)
Animals per test substance group:	8
Animals per control group:	Tap water control group: 8 Low water and low food control group: 6 The latter group received only 10 g food and 10 mL tap water per day. This treatment began in the second week after the high-dose group exhibited a decreased water- and foodintake.
Administration:	The test substance was prepared as solution in tap water. 35 mL of the test substance preparations were provided in water bottles per day and animal
Mating:	Female rats at the proeatrus stage were housed for one night with fertile males. Fertilisation was assumed by the presence of spermatozoa in the vaginal smear. That day was designated as gestation day 1.

5. Observations/analyses:

Analyses of test material preparations:	Not reported
Measurements:	Enzymatic activity of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, malic dehydrogenase
Mortality:	Not reported
Clinical signs:	Not reported
Maternal body weight:	Measured daily
Food- and water consumptions:	Measured daily
Test substance intake:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported

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Sacrifice/pathology: On day 21 of gestation, rats were anesthetisied with diethylether. Each foetus was delivered by rapid hysterectomy, identified, weighed and then killed by decapitation. Maternal and foetal livers, hearts, and brains were immediately removed, washed in a cold saline solution, and stored at -20°C until analysis. Foetal organs were pooled.

Tissue sample processing: Livers, hearts, and brains (0.5g/1 ml buffer) were homogenised in an Ultra Turrax with 0.5 M Tris-HClbuffer, pH 7.4 containing 1 mM dithiothreitol. Cytosolic fractions were obtained by ultra centrifugation.

Measurements (enzymatic assays): Enzymatic activities of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and maic dehydrogenase were measured in the supernant by the determination of the rate of NADPH formation at 340 nm in a spectrometer. The results were expressed as µmol NADP/min/mg protein. Protein concentration was measured by Biuret reaction.

Organ weights: Liver, hearts and brains of maternal females

Histopathology: Not done.

Statistics: Signifficant differences among means were considered at a level of $P < 0.05$ and identified by one-way ANOVA, Kolmogorov-Smirnov, and Newman-Keul procedures. In all the cases the variances were homogeneous.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Basic data given, however, the study is performed with methodological and reporting deficiencies (unknown exposure levels, only cytosolic enzymes measured, inappropriate controls, lack of consistent dose-response data).

2. Relevance of study: **Not relevant** (Due to reliability. In addition, study was performed with a glyphosate formulation (commercialised in Argentina) and not with glyphosate)

3. Klimisch code: **3**

Response 1 – GTF

- Test substance administration is poorly described, but rough calculations on approximate surfactant intake show excessively high and unrealistic exposures when compared to DART systemic parental and reproductive/developmental NOAEL values for POEA formulation surfactants.
  - For the low dose group, based on 360 g/L glyphosate solution containing 18% surfactant, 0.1 mL glyphosate (conservatively assumed to be the formulation)/mL water = 0.018 mL surfactant/mL water. Assuming water consumption of 10 mL/day surfactant intake = 0.18 mL per rat per day. Assuming surfactant density of 1 g/mL and 250 gram rat, surfactant low dose = 720 mg/kg/day.
  - Conservative high surfactant dose estimate = 1440 mg/kg/day
  - Conservative estimate of surfactant intake is at least one order of magnitude greater than parental and DART NOAEL values reported in Williams et al. (2012).

Response 2 – summarized from Williams et al. (2012)

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- Test substance and doses not adequately described.
- Inappropriate control groups.
- Results suggest that the effect of treatment on body and organ weights may be due to reduced food and water intakes.
- A consistent effect of treatment was not observed and dose-response relationships were generally lacking
- The information gathered may be misleading because the enzymes monitored are found in both the cytosol and mitochondria.
- Food restriction affects the activity of many enzymes, including those examined in this study.
- Same comments apply to Bueret et al. (2005; on-line version 2004), in which test group dams showed a 23% reduction in food consumption, 21% reduction in water consumption and 42% reduction in body weight gain versus controls.

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Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Coelho, R. S. Pereira, J. D. Dalsenter, P. R. Langeloh, A.	2003	The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats Toxicology letters Volume: 142 Pages: 45-52

Abstract*

The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup(R) (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Cesarean sections were performed on day 21 of pregnancy, and number of corpora lutea, implantation sites, living and dead fetuses, and resorptions were recorded. Weight and gender of the fetuses were determined, and fetuses were examined for external malformations and skeletal alterations. The organs of the dams were removed and weighed. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup(R) is toxic to the dams and induces developmental retardation of the fetal skeleton.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Roundup ®
Active substance: Glyphosate
Source: Monsanto of Brasil
Lot/Batch #: BS 1096/98
Concentration: 360 g/L
Surfactant Class: Polyoxyethyleneamine (POEA)
Concentration: 18% (w/v) (POEA)

2. Vehicle: Distilled water

3. Test animals:

Species: Rat
Strain: Wistar
Source: Department of Pharmacology, Instituto de Ciencias Basicas da Saude, Brazil
Age of test animals at study initiation: 90 days
Sex: Male and virgin female
Body weight: 200-280 g
Acclimation period: Not reported
Diet/Food: Laboratory rat chow, *ad libitum*
Water: Water , *ad libitum*

Housing:	Polyethylene (65 x 25 x 15 cm) home cages, with sawdust-covered floors
Environmental conditions:	Temperature: 22 ± 2°C Humidity: not reported Air changes: not reported 12-hour light/dark cycle

4. Test system:

Study type:	Developmental toxicity study
Guideline:	Refers to the EPA (Environmental Protection Agency), 1996. Guidelines for Reproductive Toxicity Risk Assessment-EPA/630/R-96/009, Washington, USA, pp. 1-163, (reproductive toxicity protocols, segment II).
GLP:	no
Guideline deviations:	Reduced allowed mating time
Duration of study:	From day 6 up to 15 of gestation
Dose levels:	0 (water), 500, 750 or 1000 mg/kg glyphosate-Roundup® diluted in water
Animals per dose group	Sixty pregnant rats were divided into four groups (n=15±1 per group).
Administration:	Test substance preparations were prepared by diluting the Roundup-formulation with appropriate volumes of distilled water. Applications were done once daily by oral gavage Dosing volume: 10 mL/kg bw
Mating:	3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.

5. Observations/analyses:

Test substance preparations:	Not reported
Mortality:	Assessed, but details (e.g. time points, etc) not specified.
Clinical signs:	Not reported
Body weight:	Maternal body weights were determined daily during pregnancy and lactation periods. Offspring body weights were determined in weekly intervals from lactation to puberty
Body weight gain:	The body weight noted at day 0 (sperm positive smear) in parent females was considered as 100 %. The differences observed during the study with regard to this parameter were expressed as relative weight gain.
Food- and water consumptions:	In three day intervals during pregnancy. Data presented as relative intakes without reference to how data were normalized.
Test substance intake:	Not applicable
Sacrifice/pathology:	On day 21 of gestation dams were anesthetized with a combination of 5 mg/kg bw xylazine and 90 ,g/kg bw ketamine injected intramusculary and subjected to caesarean section. The uterus was removed and weighed with its contents.

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Organ weights: The weights of the following organs were determined and relative organ-to-body weights were calculated.

Maternal: heart, lungs, liver, spleen and kidney

Developmental parameters: Number of living and death foetuses, number of implantation sites, corpora lutea, resorptionssex of pups, sex-ratio, external malformations and skeletal alterations.

Reported errors include more foetuses than implantation sites in one dose group.

Note artifacts from atypical fixing and staining of foetal skeletons may have caused skeletal damage.

Statistics: Parametric data, expressed as mean ± S.E.M., were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Duncan test when appropriate. The non-parametric data, expressed as proportion or percentage, were analyzed by the x^2-test. Differences were considered to be statistically significant when $P<0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design similar to US-EPA and OECD 414, with deviations (e.g. group size, inadequate dosing period) and reporting deficiencies. In addition, some methodological deficiencies (e.g. histopathological methods)

2. Relevance of study: Relevant study type for investigating developmental endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product, not glyphosate.

3. Klimisch code: **3**

Response 1 – GTF

- This non-guideline prenatal developmental toxicity study with a POEA containing formulation may be compared directly with the test guideline and GLP compliant POEA rat prenatal developmental toxicity study, in which the same POEA surfactant maternal NOAEL was 15 mg/kg/day, and developmental NOAEL was considered the highest dose tested, 300 mg/kg/day.
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
  - If based on dose levels of 500, 750 or 1000 mg/kg formulation, surfactant doses are 90, 135 and 180 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
  - If based on dose levels of 500, 750 or 1000 mg/kg glyphosate technical acid (versus the salt form in the formulated product), surfactant doses are even more extreme, approximately 250, 375 and 5000 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
- This publication reports excessively high and unrealistic exposures to the POEA surfactant in the tested formulation.
- While reporting weight gain in an atypical manner as relative %, actual reported mean body weight gains for mid and high dose groups align with the control group, while the low dose group body weight gain is approximately 20% less than the control group, indicating significant maternal toxicity in the low dose group. This significant non-dose related toxicity brings the quality and accuracy of this study into question.

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Response 2 – summarized from Williams et al. (2012)

- Non-guideline prenatal developmental toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- 15 rats per group, significantly lower than the recommended minimum of 20 litters per group in OECD 414.
- High dose group was further reduced to 7 pregnant dams due to maternal deaths.
- Few data presented in the publication.
- Unusual data presentation for body weight, food intake and water consumption, all a relative numbers without any reference to normal values.
- Fetal findings are presented as percentages or unsubstantiated mean values throughout the article, which complicates interpretation.
- Further investigation data presented notes a number of reporting errors (see Williams et al., 2012, Table 3). For example, in the 750-mg/kg/d treatment group, more fetuses than implantation sites were reported.
- Reports a dose-related increased incidence of skeletal alterations.
- Unusual methods described to fix and stain the fetal skeletons for evaluation, which may have led to artifacts that were falsely categorized as alterations (use of a a proteolytic enzyme which may have digested peptide bonds in the bone matrix). The reported skeletal alterations showed an extremely high prevalence of incomplete ossification of various bone structures, which are signs of a developmental delay that correct themselves within a brief period.
- treatment during gestation days 6-15 rather that to full term as per current test OECD 414 guidelines
- "Based on the use of these questionable methods, and the obviously flawed reporting of data, it is not possible to draw any conclusions regarding the developmental effects of "Roundup" treatment from this article. Furthermore, because a commercial formulation was used, it is not possible to attribute any observed effects to glyphosate specifically."

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Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Oliveira, R. T. Andrade, A. J. M. Dalsenter, P. R. Langeloh, A.	2007	Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats Archives of Toxicology Volume: 81 Pages: 665-673

Abstract*

Glyphosate is the active ingredient and polyoxyethyleneamine is the surfactant present in the herbicide Roundup (R) formulation commercialized in Brazil. The aim of this study was to assess the reproductive effects of glyphosate-Roundup (R) on male and female offspring of Wistar rats exposed during pregnancy and lactation. Dams were treated orally with water or 50, 150 or 450 mg/kg glyphosate during pregnancy (21-23 days) and lactation (21 days). These doses do not correspond to human exposure levels. The results showed that glyphosate-Roundup (R) did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup (R) may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup®
Active substance(s):	Glyphosate
Source:	Monsanto of Brazil
Lot/Batch #:	Not reported
Concentration:	360 g/L
Surfactant:	Polyoxyethyleneamine (POEA)
Concentration:	18% (w/v) POEA

2. Vehicle: Distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Department of Pharmacology, Federal University of Rio Grande do Sul, Brazil
Age of test animals at study initiation:	90 days
Sex:	Male and female
Body weight:	250-350 g
Acclimation period:	Not reported
Diet/Food:	standard lab rat chow (Nuvital®, Curitiba/PR, Brazil), *ad*

libitum

Water: Water, *ad libitum*

Housing:: Polyethylene (65 x 25 x 15 cm) home cages with sawdust-covered floors

Environmental conditions: Temperature: 22 ± 2°C
Humidity: not reported
Air changes: not reported
12-hour light/dark cycle

4. Test system:

Study type: Reproductive toxicity

Guideline: None

GLP: No

Guideline deviations: Not applicable

Duration of study: 21-23 days during pregnancy;
21 days during lactation

Dose levels: 0 (water), 50, 150, 450 mg/kg glyphosate-Roundup®

Mating: 3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.

Animals per dose group: Sixty primigravid female rats were randomly divided into 4 groups of 15 animals each.

Administration: Test substance preparations were prepared by diluting the Roundup-formulation with appropriate volumes of distilled water.
Applications were done once daily by oral gavage
Dosing volume: 10 mL/kg bw

5. Observations/analyses:

Test substance preparations: Not reported

Mortality: Assessed, but details (e.g. timepoints, etc) not specified.

Clinical signs: Assessed, but details (e.g. timepoints, etc) not specified.

Body weight: Maternal body weights were determined daily during pregnancy and lactation periods.
Offspring body weights were determined in weekly intervals from lactation to puberty.

Body weight gain: The body weight noted at day 0 (first period day) in parent females was considered as 100 %, for each period. The differences observed during the study with regard to this parameter were expressed as relative weight gain.

Food- and water consumptions: Not done

Test substance intake: Not applicable

Haematology: Not done

Clinical chemistry: Not done

Hormone levels: For determination of testosterone levels, blood was collected at termination, and the serum was removed. The samples were

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analysed in duplicate using a double-antibody according to the standard protocol for the radioimmunoassay (RIA) with Diagnostic Products Corporation testosterone kits.

Urine analysis: Not done

Litter data: Litter size, number of living and dead pups, viable pups, sex ratio (male/female)

Offspring development: The development of offspring was assessed daily from lactation until puberty. The following characteristics were assessed: ears unstuck, fur emergence, incisor eruption, eye opening, testis descent (by scrotum palpation starting after the 15th postnatal day), preputial separation (by manually retracting the prepuce with gentle pressure after the 30th postnatal day) and opening of the vaginal canal (after the 30th postnatal day)

Sacrifice/pathology: Males:

One male from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65 days) and adulthood (age: 140 days). Selected males were sacrificed by thiopental anaesthesia followed by diaphragm incision.

Females:

One female from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65-70 days) and adulthood (age: 140 days).

Organ weights: The weights of the following organs were determined and relative organ-to-body weights were calculated.
Males: heart, lungs, liver, spleen, kidneys, adrenal glands and brain; testis, epididymis, seminal vesicle with coagulating glands (without fluid) and prostate
Females: heart, lungs, liver, spleen, kidneys, adrenal glands and brain; uterus, oviducts and ovaries

Histopathology: Five testes per dose group were fixed in Bouin´s solution immediately after removal, embedded in paraffin, sectioned at 3 µm and stained with hematoxylin/eosin.

20 essentially round seminiferus tubules per testis were analysed microscopically. The following parameters were assessed: tubule diameter, percentage of seminiferus tubules with complete spermatogenesis, presence of degenerating, sloughed and/or infiltrating cells, and absence of tubular lumen and of elongated spermatids.

Reproductive toxicity assessment: Relative weight of the reproductive organs expressed as percentage of body weight and of reproductive indices, including sperm number per epididymis tail, daily sperm production, sperm transit, sperm morphology, testis morphology and serum testosterone level. Spermatid and sperm counts were determined.

Statistics: Parametric data, expressed as mean ± standard error (SEM), were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Bonferroni test when appropriate. The nonparametric data, expressed as proportion or percentage, were analyzed by the chi-square test. Differences were considered statistically significant when $P < 0.05$.

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Study that does not comply with any test guideline. Reporting deficiencies. Conflicting results include decreased testes weights but increased testosterone levels in high dose. Questionable micrograph quality and interpretation may be artifacts of processing techniques. Conclusions not consistent with findings when viewed in light of dose-response or historical data for this strain of rat.

2. Relevance of study: **Not relevant** based on lack of dose-response, contradicting findings and unreliable data quality)

3. Klimisch code: **3**

Response 1 – GTF

- This non-guideline reproductive toxicity study with a POEA containing formulation may be compared to the DART NOAEL values for POEA surfactants reported in Williams et al. (2012).
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
- If based on dose levels of 50, 150 or 450 mg/kg formulation (18% POEA), surfactant doses are 9, 27 and 81 mg/kg/day. In this case, doses are in the range of NOAEL values reported by Williams et al. (2012).
- Based on dose levels of 50, 150 or 450 mg/kg glyphosate technical acid (versus the salt form in the formulated product), POEA surfactant doses would be approximately 25, 75 and 225 mg/kg/day. In this case the low/mid doses are in the range of NOAEL values and the high dose exceeds NOAEL values reported by Williams et al. (2012).
- The findings reported by Dallegrave et al. (2007) are contrary to the GLP and guideline compliant studies reviewed by Williams et al. (2012), in which no effects on testis morphology, sperm parameters or testosterone levels were evident.

Response 2 - summarized from Williams et al. (2012)

- Non-guideline prenatal developmental-reproductive toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- Maternal toxicity was not observed.
- Reproductive outcomes (number of pups, sex ratio, etc.) and pup weights unaffected.
- Statistical increased percentage of abnormal sperm in male offspring at the low but not medium or high dose offspring, suggesting a random finding
- Non-dose-related delay in vaginal opening in females within the normal physiological range for the species and in line with historical control data.
- Non-dose-related early preputial separation in the high dose males within the normal physiological range for the species and in line with historical control data.
- Contrary to expected outcome of early preputial separation, a statistical decrease in blood testosterone levels was also observed at puberty for high dose males.
- Decreased testosterone level was no longer evident at adulthood
- No dose-related findings in adult sperm production parameters
- Investigators fail to mention enlarged interstitial cells in the micrographs, suggesting limited experience conducting such histological examinations.
- The other reported histological interpretations, reduction in elongated spermatids and the presence of vacuolization at puberty and degeneration of the tubular lumen at adulthood, may be attributable to an artifact of tissue processing rather than exposure related effects.

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- Multiple guideline study types and a subchrionic National Toxicology Program study do not report the testicular anomalies described by Dallegrave et al. (2007).

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Author(s)	Year	Study title
Romano, R.M. Romano, M.A. Bernardi, M.M. Furtado, P.V. Oliveira, C.A.	2010	Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Archives of Toxicology Volume: 84 Pages: 309-317

Abstract*

Glyphosate is a herbicide widely used to kill weeds both in agricultural and non-agricultural landscapes. Its reproductive toxicity is related to the inhibition of a StAR protein and an aromatase enzyme, which causes an in vitro reduction in testosterone and estradiol synthesis. Studies in vivo about this herbicide effects in prepubertal Wistar rats reproductive development were not performed at this moment. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis. Results showed that the herbicide (1) significantly changed the progression of puberty in a dose-dependent manner; (2) reduced the testosterone production, in semineferous tubules' morphology, decreased significantly the epithelium height ($P < 0.001$; control = 85.8 ± 2.8 µm; 5 mg/kg = 71.9 ± 5.3 µm; 50 mg/kg = 69.1 ± 1.7 µm; 250 mg/kg = 65.2 ± 1.3 µm) and increased the luminal diameter ($P < 0.01$; control = 94.0 ± 5.7 µm; 5 mg/kg = 116.6 ± 6.6 µm; 50 mg/kg = 114.3 ± 3.1 µm; 250 mg/kg = 130.3 ± 4.8 µm); (4) no difference in tubular diameter was observed; and (5) relative to the controls, no differences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 ± 12.9 ng/dL; 5 mg/kg = 108.6 ± 19.6 ng/dL; 50 mg/dL = 84.5 ± 12.2 ng/dL; 250 mg/kg = 76.9 ± 14.2 ng/dL). These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L as isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported
Age of test animals at study initiation:	21 days
Sex:	Male
No. of rats:	68
Body weight:	Not reported

Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats
Water:	Mineral water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Evaluation of endocrine disruption potential of glyphosate formulation by assessment of rats prepubertal reproductive development.
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	From postnatal day (PND) 23 until PND53
Dose levels:	Control group – deionized water; 5, 50 or 250 mg/kg of body weight of glyphosate-Roundup Transorb
Animals per dose group:	4 treatment groups, 17 animals per group
Animal selection	No mention of avoiding selection of siblings within the same group to control for possible litter effects
Administration:	The glyphosate-Roundup Transorb was diluted in a watery suspension and administered once a day, by gavage; Dosing volume: 0.25 mL/100 g of body weight, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The experimental design was composed of random blocks, with the formation factor of these blocks as the body weight at the PND23. All the animals were weighed, and the average and standard deviation were calculated. The animals having body weights lower or higher than two standard deviations from the average were removed from the experiment.
Determination of puberty age:	Evaluation of the balanopreputial separation was made, which consists of the separation of the preputial membrane and the externalization from the glands of the penis. The assessment, which included gentle tissue manipulation, was performed once per day from the PND33 and was completed at the time of the balanopreputial separation. No discussion on whether this was a blinded procedure to avoid bias.
Hormone measurements:	Hormone concentrations of testosterone, estradiol and corticosterone in the serum were measured by radioimmuno-assay (RIA) from commercial kits (Testosterone Total Coat-A-

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Count, Estradiol Coat-A-Count and Coat-A-Count Corticosterone in rats, DPC, Los Angeles, CA, USA).

Food- and water consumptions: Not reported

Haematology: Not done

Clinical chemistry: Not done

Urine analysis: Not done

Sacrifice/pathology: On PND 53. No details reported.

Organ weights: The testes (right and left) and the adrenal glands (right and left) were weighed in absolute values and then transformed to relative weights as mg/100 g of body weight at PND53.

Histology and morphometry: The testes and adrenal glands of all 68 animals werefixed in Bouin´s solution for 8 h, treated with alcohol, embedded in paraffin and prepared as stained laminas with hematoxylin and eosin.

Laminas were analysed by light microscopy (40x and 100 x magnification).

The linear morphometry from the seminiferous tubules were analysed by determining the tubular diameter (measured from the basal lamina to the basal lamina in the opposite direction), seminiferous epithelium (from the basal lamina to the neck of the elongated spermatids) and luminal diameter. Micrographs presented are of poor quality with artifacts such as shrinkage. Considered together with the natural variably in spermatogenesis of pubescent rats, the accuracy of morphometric data comes into question.

For each tubule, the averages were calculated for the measurements indicated and, then, the average of each Weld was also calculated. The measurement for each animal was obtained through measure of all the analyzed Welds.

Statistics: The variables under study were first submitted to tests of normality from Kolmogorov–Smirnov and homocedasticity by the test of Bartlet. When some of the premises of parametric testing were not obtained, non-parametric tests were chosen for subsequent averages and tests. Statistical differences were considered significant when the value of P was lower than 0.05. The values were expressed in mean (x) and standard error of the mean (±SEM).

Data analysis of daily weights was performed through the two-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM). The weights were compared between diVerent groups and diVerent ages, considering the evolution expected by the body growth. The day and the weight of the complete balanoprepucial separation were compared among the groups using non-parametric analyses by the Kruskal–Wallis method followed by the post hoc Dun test. The testis and the adrenal weights were analyzed by the Kruskal–Wallis followed by the post hoc Dunn test, or by using a one-way analysis of variance (ANOVA) followed by the post hoc Tukey test. The testis measures of tubular diameter and epithelium depth, as well as the serum concentrations of testosterone, estradiol and corticosterone, were analyzed by the ANOVA followed by the Tukey test.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study with methodological and reporting deficiencies or conflicting findings. Eg, increased relative testicular weights, but decreased testosterone measurements.

2. Relevance of study: Relevant study type for investigating male reproductive endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product, not glyphosate.

3. Klimisch code: **3**

A comprehensive review, pointing out a significant number of issues with this publication, was undertaken by experts in reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc L and included in Appendix K and their summary is quoted below.

> "To the uninformed reader, this manuscript by Romano et al. appears to demonstrate that exposure to Roundup Transorb alters testosterone levels and testis morphology. In this respect, the importance of these data to the scientific literature can be grossly over-interpreted by the uninformed reader. Upon closer examination, the authors have failed to provide robust data to support their conclusion that the "commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats". The authors failed to measure many of the key parameters in the validated pubertal male assay protocol by Stoker et al., (2000a) and hence generated data that were internally inconsistent, incomplete or in error. The results lack the scientific rigor necessary to support a definitive scientific conclusion and certainly do not equal or offset previous large, definitive and GLP-compliant studies concluding that Roundup and glyphosate do not affect reproductive development."

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Author(s)	Year	Study title
Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., de Souza, P.B., Viau, P., Bernardi, M.M., Nunes, M.T., de Oliviera, C.A.	2012	Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression Archives of Toxicology Volume: 86 Number: 4 Pages: 663-673

Abstract*

Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-day-old male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount; testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate (isopropylamine salt)
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported
Age of test animals at study initiation:	90 days
Sex:	Female

No. of rats:	12
Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats, *ad libitum*
Water:	Water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Glyphosate effects on the reproductive development of male offspring
Guideline:	Non-guideline study
GLP:	No
Guideline deviations:	Not applicable
Duration of exposure:	From gestational day 18 to postnatal day (PND) 5
Dose levels:	Control group – deionised water; 50 mg/kg bw of glyphosate
Animals per dose group:	2 treatment groups, animals per group – not reported
Administration:	Roundup Transorb was diluted in a watery suspension and administered once a day by gavage from Gestation Day 18 to Post Natal day 5; Dosing volume: 0.25 mL/100 g bw, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The pups were weighted at PND21 (weaning), PND30, PND40 and PND60 to compare the body growth between the groups.
Sexual partner preference:	The sexual partner preference was assessed at PND 60 by exposing male offspring from treated and non-treated mothers to female stimulus rats (i.e. ovariectomised female rats that were treated with estradiol (50 µg/kg bw s.a. 54 h before the test) and progesterone (2 mg/kg bw s.c., 6 h before test)).
Sexual behaviour:	Sexual behaviour was assessed at PND 60 by exposing the male rats to an oestrus-induced female for 40 min. Several parameters were assessed incl. Number of mounts, intromission, ejaculatory intervals, number of attempted mounts).
Determination of puberty age:	Evaluation of the balanopreputial separation (separation of the preputial membrane and externalization from the glands of the penis). The assessment (including gentle tissue manipulation) was

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performed once per day from the PND33 and was completed at the time of the balanopreputial separation.

Hormone measurements: Hormone serum concentrations of testosterone, estradiol in the serum were measured by radioimmunoassay-assay (RIA) from commercial kits (Coat-A-Count, DPC, Los Angeles, CA, USA).
The serum FSH and LH measurements were determined by chemiluminescence immunoassay using Luminex xMAP technology (Milliplex MAP rat pituitary panel, Billerica, MA, USA).

Pituitary hormone levels: mRNA-levels of LH, FSH and GH were assessed by real-time PCR in homogenised pituitary tissues. Protein expression of LH, FSH and GH was assessed in homogenised pituitary tissues using Western-blot analysis.

Food- and water consumptions: Not reported

Haematology: Not reported

Clinical chemistry: Not reported

Urine analysis: Not reported

Sacrifice/pathology: Not reported

Organ weights: The testes, epididymides and seminal vesicle were weighed, and the values were converted to relative weights of mg/100 g bw at PND60. The epididymis was previously divided into three segments: caput, corpus and cauda. The seminal vesicle was weighted with fluid (undrained) and after fluid removal (drained).

Sperm evaluation: At PND 60, the sperm counts were determined. Testes and epididymes (caput, corpus, cauda) were weighed. The tunica albuginea was removed from the testes, and the parenchyma was homogenized. The samples were then diluted 10 times in saline, and the mature spermatids resistant to homogenization were counted using a hemocytometer. Daily sperm production was calculated.
The segments of the epididymis were cut with a scissor, homogenized, diluted and counted. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for the parts of the epididymis were calculated. The mean time for sperm transit through the epididymis was calculated.

Histology and morphometry: The testes were fixed in Bouin's solution for 8 h, treated with alcohol and embedded in paraffin, and were prepared as stained laminas with hematoxylin and eosin.
Laminas were analysed by light microscopy (40x and 100 x magnification).
Linear morphometry of the seminiferous tubules were analyzed by determining the tubular diameter, seminiferous epithelium length and luminal diameter.
For each tubule, the averages were calculated for the measurements indicated, and the average of each field was also calculated. The measurement for each animal was obtained by measuring all the analyzed fields.

Statistics: First the Kolmogorov–Smirnov tests for normality and the Bartlett test for homoscedasticity. For analysis of body growth the multi-way analysis of variance for repeated measures

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(MANOVA) by a general linear model (GLM) was used. Weights were compared between different groups and ages, considering the expected changes with age. The sexual behavior and day of PPS were compared among the groups using the Mann–Whitney *U* test. Weights of seminal vesicle (drained and undrained) were compared by paired Student's *t-test.* All other parameters were analyzed by Student's *t-test*. Statistical differences were considered significant when the value of *P* was < 0.05. Values were expressed as means and the standard error of the mean (±SEM) for parametric and interquartile ranges of nonparametric analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline, non-GLP study meeting scientific principles. Unusual and short dosing regiment commencing towards the end of pregnancy (GD18, rather than GD6 as per OECD Test Guidelines 414) through post natal day 5. *In vivo* study with reporting deficiencies (detailed strain description, source of animals, housing conditions, no information if clinical signs were assessed, stability and homogeneity assessment of test substance preparations, no of male offspring evaluated in individual tests evaluated). A number of atypical endpoints evaluated.

2. Relevance of study: Not relevant (due to questionable dosing regimen and atypical array of endpoints measured

3. Klimisch code: **3**

This quality and value of this follow up study to Romano et al. (2010) is consistent with their previous publication. Selective literature citations in the introduction frame the basis for this research as endocrine disruption potential, referring mostly to the publications from Seralini laboratory, previously discussed. The concluding sentences inaccurately cite published *in vitro* research (Richard et al., 2005) as evidence that "women occupationally exposed to this herbicide have reproductive disorders".

From the outset, the study design and endpoint selection are not consistent with other research in the field of developmental and reproductive toxicology, suggesting a lack of experience in this well published and studied discipline. Dosing was very limited to dams, starting on gestation day 18, well after organogenisis, through post natal day 5. Any glyphosate exposure to offspring either before or after parturition is questionable. ADME studies with glyphosate clearly demonstrate poor absorbtion via the gastrointestinal tract, rapid excretion of systemic doses via urine and a lack of bioaccumulation. Restricted placental transfer for glyphosate is documented in an *ex vivo* human perfusion system, in which the three other compounds tested (caffeine, benzoic acid and antipyrine) demonstrated much greater transfer kinetics across the placenta (Mose et al., 2008). Given the physico-chemical properties and *in vivo* kinetics of glyphosate, exposure to offspring during lactation should be considered negligible, if any.

With the very short window of maternal exposure, biological plausibility of any test substance related effects in the mature offspring is questionable. However, the normal variability of some unusual or atypical endpoint measurements, such as "sexual partner preference" along with mRNA and protein expression, is not known. Therefore, results are difficult to interpret, particularly for relevance to human health risk assessment. The merits of this publication should be placed in context with the quality of the

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authors' previous published research (Romano et al., 2010), as critiqued by experts in DART and ED above.

IIA 8.17 Summary and evaluation of points IIA 7 and IIA 8.1 to 8.17

IIA 8.17.1 Overall summary: IIA7, Fate and behaviour in the environment

Relevant residues of glyphosate in soil

Glyphosate is degraded in soil over time to a single major metabolite aminomethylphosphonic acid (AMPA). Several other minor components were also detected but none were present in amounts greater than 3% of the applied glyphosate. The maximum amount of AMPA detected in aerobic soil metabolism studies ranged from 14.7 to 50.1% of the total glyphosate applied. These studies clearly demonstrate that AMPA is further degraded by soil micro-flora, although at a slower rate than glyphosate. They also show that 23.6 to 79.6% of the applied glyphosate is mineralised to carbon dioxide.

Relevant residues of glyphosate in water

In addition to microbial degradation, a major contributor to the aquatic dissipation of glyphosate is adsorption to the sediment. Approximately 6 to 48% of the applied glyphosate is mineralized to carbon dioxide during 100 or 120 days of incubations. The principal metabolite of glyphosate in water/sediment system is AMPA. The maximum amount of AMPA ranges from 2 to 16% (water phase) and up to 27% (total system) of the total glyphosate applied. AMPA quickly dissipates from the water phase by both adsorption to the sediment and by degradation by the sediment micro-flora. Studies demonstrated that from 8 to 40% of the applied AMPA is mineralized to carbon dioxide. In addition, hydroxymethylphosphonic acid (HMPA) was detected in the water phase of one of the studies evaluated in IIA 7.8 with maximum amount of about 10% of the dose after 61 days.

Relevant residues of glyphosate in air

Glyphosate has a very low vapour pressure (1.31×10^{-5} Pa at 25°C) and significant concentrations are not expected to be found in air following the use of the compound according to the proposed GAP.

As the expected distribution to the atmosphere by glyphosate is likely to be extremely low in field use based on very low vapour pressure, no estimates of environmental concentrations expected in air were provided. This is not considered a significant route of exposure in practice or likely to lead to significant environmental contamination.

IIA 8.17.2 Overall summary: IIA8.1 – 8.16, Ecotoxicological Studies

Effects on birds

Test Type	Test Substance	Ecotoxicological endpoint
Acute toxicity to birds	Glyphosate acid	LD_{50} = 4334 mg a.s./kg bw (extrapolated endpoint from LD_{50} > 2000 mg a.s./kg bw)
Reproductive toxicity to birds	Glyphosate acid	NOEC = 201 mg a.s./kg bw/day (Bobwhite quail, 20 weeks one generation reproduction)

Effects on aquatic organisms

Test Type	Test Substance	Ecotoxicological endpoint
Acute toxicity to fish	Glyphosate acid	LC_{50} = 38 mg a.s./L (Rainbow trout,

		96 h)
Chronic toxicity to fish	Glyphosate acid	NOEC = 25.7 mg a.s./L (Fathead minnow, full life cycle test, flow-through, 255 d)
Bioconcentration	Glyphosate acid	BCF = 1.1 ± 0.61; steady state reached after 120 ± 59 d (Bluegill sunfish, flow through, 56 d)
Acute toxicity to aquatic invertebrates	Glyphosate acid	EC_{50} = 40 mg a.s./L (*Daphnia magna*, 48 h)
Chronic toxicity to aquatic invertebrates	Glyphosate acid	NOEC = 30 mg a.s./L (*Daphnia magna*, semi-static, 21 d)
Effect on algal growth	Glyphosate acid	E_bC_{50}=4.47 mg a.s./L (*Nitzschia palea*, static conditions, 96 h)
Effects on sediment dwelling organisms (*Chironomus riparius*)		Study not triggered
Effects on sediment dwelling organisms - Chronic test		Study not triggered
Effects on aquatic plants	Glyphosate acid	$EC_{50, \text{fresh weight, relative increase}}$ = **12.3**

Effects on honeybees

Test Type	Test Substance	Ecotoxicological endpoint
Acute oral toxicity	Glyphosate acid	LD_{50} (48 h): >200 µg/bee
Acute contact toxicity	Glyphosate acid	LD_{50} (48 h): > 200 µg/bee
Bee brood feeding test	Glyphosate acid [1]	NOAEL 266 mg/kg

[1] Test as the IPA salt of glyphosate

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Effects on other arthropod species

Test Species	Test Substance	Exposure	Application Rate	Ecotoxicological endpoint
Parasitoids				
Aphidius rhopalosiphi	MON 52276[1]	Lab, inert substrate	10 L pr./ha	100% mortality
Aphidius rhopalosiphi	MON 52276[1]	Ext. lab., barley plants	4, 6, 8, 12, and 16 L pr./ha	LD_{50}> 16 L pr./ha i.e. LD_{50}> 5760 g a.e./ha No reduction of reproduction
Predatory mites				
Typhlodromus pyri	MON 52276[1]	Lab, inert substrate	10 L pr./ha	100% mortality
Typhlodromus pyri	MON 52276[1]	Ext. lab., vine leaf discs	3, 6 and 12 L pr./ha	36% mortality at 3 L/ha 86% mortality at 6 L/ha 88% mortality at 12 L/ha No effects on fecundity at 3 L/ha 10% fecundity at 6 L/ha
Typhlodromus pyri	MON 52276[1]	Ext. lab., whole bean leafs	0.6, 3, 6 and 12 L pr./ha	LD_{50}> 16 L pr./ha No mortality at 0.6 L/ha 21% mortality at 3 L/ha 36% mortality at 6 L/ha 30% mortality at 12 L/ha No effects on fecundity at 12 L/ha or less
Foliage-dwelling predators				
Chrysoperla carnea	MON 52276[1]	Lab, inert substrate	0.6, 3, 6 and 12 L pr./ha	LD_{50}> 12 L pr./ha 59% mortality and 20% reduction on fecundity at 12 L/ha (no dose-response effect on fecundity); No effects on survival or fecundity at 0.6 and 6 L/ha
Soil-dwelling predators				
Poecilus cupreus	MON 52276[1]	Lab, inert substrate	10 L pr./ha	LD_{50}> 10 L pr./ha No effects on survival or feeding activity
Pardosa ssp.	MON 52276[1]	Lab, inert substrate	10 L pr./ha	LD_{50}> 10 L pr./ha No effects on survival or feeding activity
Trechus quadristriatus	MON 52276[1]	Lab, inert substrate	3.6 kg a.s./ha	LD_{50}>3.6 kg a.s./ha 14% adult mortality
Bembidion lampros	MON 52276[1]	Semi-field	4.89 kg a.s./ha	LD_{50}> 4.89 kg a.s./ha 0% adult mortality

[1] Tested as the IPA salt and applied as MON 52276

Effects on earthworms

Test Type	Test Substance	Ecotoxicological endpoint
Acute toxicity	Glyphosate acid	LC_{50} (14 d) > 1000 mg/kg dry soil
Reproductive toxicity	Glyphosate acid [1]	NOEC = 472.8 /kg

[1] Tested as the IPA salt of glyphosate

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Effects on soil micro-organisms

Test Type	Test Substance	Ecotoxicological endpoint
Nitrogen mineralization	Glyphosate acid [1]	No significant effects (>25%) on nitrogen mineralisation at 18.8 and 94 mg MON 57726/kg dry soil (12 and 60 L MON 52276/ha). Deviations compared to the control within the test period of 28 days are <25% and thus meet the trigger of the OECD-guidelines.
Carbon mineralization	Glyphosate acid [1]	No significant effects (>25%) on carbon transformation at 18.8 and 94 mg MON 57726/kg dry soil (12 and 60 L MON 52276/ha). Deviations compared to the control within the test period of 28 days are <25% and thus meet the trigger of the OECD-guidelines.
Nitrogen mineralization	AMPA	No significant effects (>25%) on nitrogen mineralisation at concentrations of up to 160 mg/kg dry soil. Deviations compared to the control within the test period of 28 days are <25% and thus meet the trigger of the OECD-guidelines.
Carbon mineralization	AMPA	No significant effects (>25%) on carbon transformation at concentrations of up to 160 mg/kg dry soil. Deviations compared to the control within the test period of 28 days are <25% and thus meet the trigger of the OECD-guidelines.

[1] Tested as the IPA salt and applied as MON 52276

Marine or estuarine organisms

Acute toxicity	No studies triggered

Effects on non-target plants

Vegetative vigour	ER_{50} (visual damage) = 140 g a.s./ha for Oilseed rape (*Brassica napus)* for glyphosate acid WP formulation.
Seedling emergence	no adverse effects (i.e. greater than 50%) on Purple nutsedge (*Cyperus rotundus*), Oat (*Avena sativa*), Winter wheat (*Triticum aestivum*), Maize (*Zea mays*), Onion (*Allium cepa),* Sugar beet (*Beta vulgaris*), Lettuce (*Lactuca sativa*), Oilseed rape (*Brassica napus*), Cucumber (*Cucumis sativa*), Soybean (*Glycine max),* Okra (*Abelmoschus esculentus*) and Rhubarb (*Rheum rhoponticum*) by an application of 4.48 kg a.s/ha applied as glyphosate acid WP formulation.

Effects on other non-target organisms believed to be at risk

Test Species	Test Substance	Ecotoxicological endpoint
Folsomia candida	IPA salt of glyphosate	EC_{50} (14 d) >587 mg glyphosate acid equivalents./kg dry soil
Hypoaspis aculeifer	IPA salt of glyphosate	EC_{50} (28 d) >472.8 mg glyphosate acid equivalents /kg dry soil

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Effects on biological methods for sewage treatment

Test System	Test Substance	Ecotoxicological endpoint
Inhibition of respiration rate of the activated sludge	Glyphosate acid	EC_{50} > 1000 mg /L

Justified proposals for the classification and labelling of the active substance according to Regulation (EC) No 1272/2008.

With respect to ecotoxicological data:

Acute fish toxicity (Rainbow trout):	96-hour LC_{50} = 38.0 mg a.s./L
Chronic fish toxicity (Zebra fish):	168-hour sac-fry exposure NOEC = 3.2 mg a.s./L
Acute toxicity invertebrates (*Daphnia magna*):	48-hour EC_{50} = 40.0 mg a.s./L
Chronic toxicity invertebrates (*Daphnia magna*):	21-day NOEC = 30 mg a.s./L
Algae (*Nitzschia palea*):	96-hour E_bC_{50} = 4.47 mg a.s./L
	96-hour NOEC = 1.0 mg a.s./L
Aquatic macrophyte (*Lemna gibba*)	14-day EC_{50} = 12.0 mg a.s./L
	14-day NOEC = 3.0 mg a.s./L
Biodegradability:	Classified as not ready biodegradable (see IIA 7.7)

CLP classification and labelling

Based on Commission Regulation 790/2009 (amending EC regulation 1272/2008 (CLP))

Hazard Symbol(s):		
Classification:	Aquatic Chronic 2	
Signal word(s):	none	
Hazard statement:	H411	Toxic to aquatic life with long lasting effects
Precautionary statement:	P273	Avoid release to the environment
	P391	Collect spillage
	P501	Dispose of contents/container in accordance with local/regional/national/international regulations.
Plant protection products:	EUH 401	To avoid risks to human health and the environment, comply with the instructions for use.

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APPENDIX I

Conversion of avian dietary study results from feed concentration to daily dose

Results of avian short-term dietary studies have been converted to daily dose (mg/kg bw) as recommended in 'Guidance of EFSA, Risk Assessment for Birds and Mammals', European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2009: 7(12):1438. A summary of the daily dose for each treatment level for the two short-term dietary exposure dietary studies with glyphosate acid is depicted below.

Toxicity of MON 52276 and glyphosate acid to birds

Study	Species	Endpoint	Result	Reference
Glyphosate acid				
Short-term dietary	Bobwhite quail	LC_{50} NOEC (mg/kg feed)	> 5200 5200 [1]	395/963857 1997
	Mallard duck	LC_{50} NOEC (mg/kg feed)	> 5200 5200 [1]	23 1997

[1] Highest dose tested.

The daily dose for birds in each treatment group of each test, expressed as test substance (TS) intake, was calculated by treatment group using the following formula:

$$\text{Test substance intake (mg TS/g bw/day)} = (\text{Consumption}_{mean} \times \text{Conc}_{Feed}) / \text{BW}_{mean}$$

$\text{Consumption}_{mean}$= Group Mean Feed Consumption (g/bird/day)
Conc_{Feed} = Concentration (mg TS/kg feed)
BW_{mean}= Group Mean Body Weight for Start of Treatment and Exposure Termination (g)

The values used in the calculations and the daily dose values are presented in the tables below.

Daily dose from glyphosate acid short-term avian studies

Dietary Dose Level (mg a.s./ kg feed) [1]	Group Mean Feed Consumption (g/bird/day)	Group Mean Body Weight (g)	Daily Dose (mg a.s./kg bw/day)
Bobwhite quail dietary study (days 1 and 5)			
Control	5.0	19.0	0
325	5.0	18.5	88
650	5.3	18.6	186
1300	4.9	18.0	354
2600	5.5	19.2	747
5200	5.2	17.9	1511
Mallard duck dietary study(days 0 and 4)			
Control	63	192.5	0
325	63	191.5	107
650	60	190	205
1300	58	179.5	420
2600	61	186	853
5200	63	191	1715

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Daily dose from glyphosate acid avian reproduction studies

Nominal Dose (mg a.s./kg feed)	Daily mean food consumption (g feed/bird/day)	Mean body weight (g)	Daily dose (mg a.s./kg bw/day)
Bobwhite quail			
Control	21.1	218.5	0
50	20.3	208.0	4.9
200	19.8	218.3	18.1
1000	20.4	211.8	96.3
Mallard duck			
Control	131.1	1154.3	0
50	118.8	1078.3	5.5
200	128.9	1135.5	22.7
1000	145.5	1161.3	125.3
Bobwhite quail[a]			
Control	18.9	226	0
500	18.9	230	41.1
1000	18.8	222	84.7
2250	19.8	222	201
Mallard duck[b]			
Control	140.6	1098	0
500	140.0	1083	64.6
1000	128.0	1093	117
2250	142.4	1069	300

[a]Syngenta study report number 123-186.
[b]Syngenta study report number 123-187.

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APPENDIX II

Appendix II provides a review and comparison of 1978, 1987c and 1996. The tests performed by 1978 and (1987c) do not meet the current OECD 201 test validity criteria for algal tests (OECD 201, 2006). However, 1996) meets the current OECD 201 validity criteria and therefore is appropriate for use in an ecological risk assessment.

A comparison of the algal assay tests with glyphosate acid against the current OECD Validity Criteria is given below.

1. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour period.

Table 1: Comparison of biomass in the algal studies

	1978	**(1987)**	**(1996)**
Day	**Mean control cell counts**		
0	20000	10000	7760
1	Data not provided	Data not collected	18800
2		16000	97200
3		36333	554000
72-h increase factor		3.6	71.4

The (1987) control cell counts increased by a factor of only 3.6 within the initial 72-hour period. A 3.6 factor is significantly below the required criterion of 16-fold growth during the 72-hour test period. However, the (1996) control cultures increased by a factor of 71.4 during the same period. Consequently, it must be concluded that there was a major issue with the algal cultures in the study that is significant enough to render the study invalid for risk assessment purposes.

2. The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.
3. The coefficient of variation of average specific growth rates during the whole test period in the replicate control cultures must not exceed 7% (*Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*) or 10% for less frequently tested species.

In the 1978 study, no cell counts are reported, therefore, it is not possible to verify validity criteria 2 and 3.
In the (1987) study, the required cell counts were not taken on day 1 of the study, and, therefore, there is no way to verify that validity criteria 2 and 3 above were met. The (1996) study had cell count data on all days of the study and therefore it could be verified that the study met these validity criteria as shown in Table 2 and 3 below.

Table 2. Section-by-section growth rate in controls

Control replicate	0-24 hour growth rate	24-48 hour growth rate	48-72 hour growth rate	% coefficient of variation[1]
A	0.80	1.71	1.54	35.8
B	0.94	1.47	1.66	27.7
C	0.93	1.63	1.89	33.5
D	0.84	1.71	1.89	37.9
E	0.89	1.71	1.75	33.5
F	0.90	1.63	1.65	30.9
				Mean = 33.2

[1]Coefficient of variation = SD/mean*100

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Table 3. Average specific growth rate for controls during the 72-hour test period

Control replicate	0-72 hour growth rate
A	1.35
B	1.36
C	1.48
D	1.48
E	1.45
F	1.39
Mean	1.42
SD	0.06
CV	**4.19**

In addition to that, in the pre-GLP ████ 1978 study, neither analytical verifications nor measurements are reported or were conducted, nor it is possible to reproduce in detail which substance/intermediate was actually tested.

Summary and Conclusions

The ████ (1978) and ████ (1987) marine algae (*Skeletonema costatum*) toxicity studies are outdated and do not meet the current OECD 201 Validity Criteria for testing algae. The relevant endpoints from the ████ study are presented in Table 4 below.

Table 4. Relevant endpoints from the ████ (1996) marine algae toxicity test

E_bC_{50} (0-72 hr)	11 mg a.s./L
E_rC_{50} (0-72 hr)	18 mg a.s./L
NOEC	1.8 mg a.s./L

References

████ 1978. Toxicity of seven test materials to the marine alga, *Skeletonema costatum*. Report number ██-78-4-031.

████ 1987. The toxicity of glyphosate technical to *Skeletonema costatum*. Study number ██-88-414.

OECD 201 (2006).OECD Guidelines for the Testing of Chemicals. Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Adopted 23 March 2006.

████ 1996. Glyphosate acid: Toxicity to the marine alga *Skeletonema costatum*. ██5684/B.

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